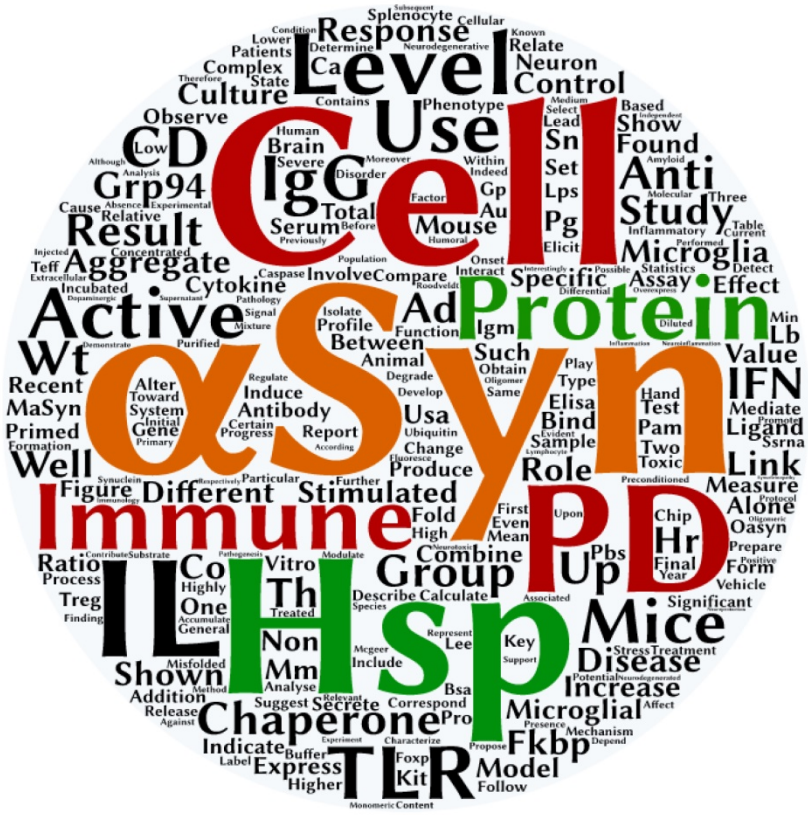
DOCTORAL THESIS

# ALPHA-SYNUCLEIN AND IMMUNE SYSTEM CROSSTALK IN PARKINSON'S DISEASE; THERAPEUTICAL APPROACHES OF ITS MODULATION BY MOLECULAR CHAPERONES

ADAHIR LABRADOR GARRIDO

Sevilla 2017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Universidad de Sevilla - Facultad de Medicina

Doctoral thesis

# Alpha-Synuclein and Immune System Crosstalk in Parkinson's Disease; Therapeutical Approaches of its Modulation by Molecular Chaperones

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## ABBREVIATIONS

| | |
|---|---|
| **AAb** | Auto antibodies |
| **AAv** | Adeno-associated virus |
| **Aβ** | Amyloid beta |
| **AD** | Alzheimer's Disease |
| **ALS** | Amyotrophic Lateral Sclerosis |
| **APC** | Antigen Presenting Cell |
| **Arg-1** | Arginase 1 |
| **aSyn** | Alpha-Synuclein |
| **AT** | Adoptive Transference |
| **AU** | Arbitrary Units |
| **BBB** | Blood Brain Barrier |
| **BSA** | Bovine Serum Albumin |
| **CNS** | Central Nervous System |
| **CpG** | Type B CpG oligonucleotide ODN1688 |
| **CSF** | Cerebrospinal Fluid |
| **DA** | Dopamine |
| **DAMPs** | Damage Associated Molecular Patterns |
| **DLB** | Dementia with Lewy Bodies |
| **EU** | Endotoxin Unit |
| **GFAP** | Glial Fibrillary Acidic Protein |
| **HD** | Huntington's Disease |
| **HSP** | Heat Shock Protein |
| **IDP** | Intrinsically Disordered Protein |

| | |
|---|---|
| **IFN** | Interferon |
| **Ig** | Immunoglobulin |
| **IL** | Interleukin |
| **Imiq** | Imiquimod |
| **iNOS** | Inducible Nitic Oxide Synthase |
| **IS** | Immune System |
| **LB** | Lewy Body |
| **LPS** | Lipopolysaccharide |
| **LTA** | Lypotheichoic Acid |
| **MaSyn** | Monomeric alpha-Synuclein |
| **MHC-I** | Mayor Histocompatibility Complex class I |
| **MHC-II** | Mayor Histocompatibility Complex class II |
| **MPTP** | 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine |
| **MSA** | Multiple System Atrophy |
| **N-aSyn** | Nitrated alpha-Synuclein |
| **NFκB** | Nuclear Factor kappa B |
| **NK** | Natural Killer |
| **OaSyn** | Oligomeric alpha-Synuclein |
| **Pam3** | Pam3 Csk4 |
| **PAMPs** | Pathogen Associated Molecular Patterns |
| **PBMCs** | Peripheral Blood Mononuclear Cells |
| **PD** | Parkinson’s Disease |
| **PET** | Positron Emission Tomography |
| **PGN** | Peptidoglycan |

| | |
|---|---|
| **PolyI:C** | Polyinosine-polycytidic Acid |
| **PRRs** | Pattern Recognition Receptors |
| **RA** | Rheumatoid Arthritis |
| **SN** | Substantia Nigra |
| **SNpc** | Substantia Nigra pars compacta |
| **ssRNA** | Single stranded Ribonucleic Acid |
| **Teff** | Effector Tcell |
| **TH** | Thyroxine Hydroxylase |
| **Th** | Helper Tcell |
| **TNF** | Tumour Necrosis Factor |
| **TLR** | Toll-like Receptor |
| **Treg** | Regulatory Tcell |
| **Wt** | Wild Type |

# 1. PARKINSON'S DISEASE

Neurodegenerative disease is a term that covers a range of conditions that primarily affect the neurons of the central nervous system (CNS). Given that neurons normally do not reproduce or replace themselves when they become damaged or die, they cannot be easily replaced by the body. Neurodegenerative diseases are incurable and debilitating conditions that result in progressive degeneration and / or death of nerve cells. This causes motor problems (called ataxias) or mental dysfunction (called dementias). In the modern world these diseases are increasing in morbidity and mortality. The World Health Organization (WHO) has predicted that by year 2030 the population suffering dementia will double, representing over 70 million people worldwide (Dementia: a public health priority, 2012(Anon 2016)). In the same way, the number of cases of Parkinson's Disease (PD) is expected to continue increasing, reaching 8.7 million people by 2030 (doubling the estimated number of cases in 2005) (Dorsey et al. 2007). Indeed, 1.2 million people were estimated to be affected by PD in 2010 in Europe and 79,000 only in Spain, representing a total estimated cost of 14,000 million and 1,800 million euros, respectively (Olesen et al. 2012; Parés-Badell et al. 2014). Moreover, Dementia with Lewy Bodies (DLB) represents ca. 20% of all the cases of dementia as determined by autopsy (McKeith et al. 2003), while Multiple System Atrophy (MSA) presents a prevalence of 4-5 cases per 100,000 people in the European Union, but about 8-10% of patients with Parkinsonism will eventually develop MSA. Therefore, a higher prevalence than that estimated can be assumed (Longo et al. 2015). Due to the increased life expectancy and population demography changes (i.e. increase in the rate of aging groups) neurodegenerative disorders are becoming increasingly more common

(Brookmeyer et al. 1998; Samii et al. 2004). Therefore, the understanding of the mechanisms involved in these disorders is becoming crucial for the development of new therapies and approaches to fight with the staggering personal, social and economic costs of these diseases.

From the increasing understanding of the pathophysiology of several of these neurodegenerative diseases during the last two decades, a common pathogenic mechanism has been pointed out: the accumulation and deposition of specific insoluble aggregates of misfolded proteins in particular regions of the brain, such as Amyloid-β (Aβ) and tau in Alzheimer Disease's (AD), Superoxide dismutase-1 (SOD1) and TDP-43 in Amyotrophic Lateral Sclerosis (ALS) or huntingtin in Huntington's Disease (HD). In the same way the so called α-synucleinopathies comprise progressive neurodegenerative diseases, including PD, DLB and MSA, with the major pathological hallmark being α-Synuclein (aSyn) inclusions in neuronal and/or glial cells. Another shared aspect of these diseases is the appearance of microgliosis, i.e. the presence of activated microglia and proinflammatory mediators, such as citokines and chemokines, in the brain of patients; initially seen in postmortem brains (Imamura et al. 2003; Mirza et al. 2000) and now extensively confirmed by Positron Emission Tomography (PET) in living patients (Gerhard et al. 2003; Gerhard et al. 2006; Iannaccone et al. 2013). Despite all the accumulating knowledge, the exact pathogenic mechanisms remain unclear. Indeed, all the accumulated data pointing to different -sometimes even opposing - directions, support the current view that these diseases are extremely complex disorders with multiple pathogenic mechanisms. And maybe that is the reason why we still lack effective therapies for these diseases.

As aforementioned, the synucleinopathies have in common the accumulation of insoluble aSyn aggregates as the main pathological feature (Marques &

Outeiro 2012). However, these inclusions appear in different locations and cell types depending on the specific disease. Cytoplasmic neuronal inclusions highly enriched in aggregated aSyn, known as Lewy bodies (LB) and dendritic or axonal Lewy neurites are key features in these diseases. Mainly present in Substantia Nigra pars compacta (SNpc) in PD and in cerebral cortex in DLB (Ferman & Boeve 2007; Spillantini et al. 1998), while the cytoplasmic aSyn filament inclusions in oligodendrocytes are characteristic in MSA (Tu et al. 1998; Wakabayashi et al. 1998). In addition, extensive aSyn staining has been detected in corticospinal axon tract fibers and glia in brain and spinal cord of ALS cases (Doherty et al. 2004), while aSyn has also been identified as a component of amyloid brain tissues in AD patients (Hamilton 2000; Hansen et al. 1990) and higher levels of non-aggregated aSyn have been measured in Cerebrospinal Fluid (CSF) of AD patients (Korff et al. 2013)

The connection between aSyn and the pathology of PD started in the late 90's when a single missense mutation in the aSyn gene, now known as A53T, was related with an autosomal dominant form of hereditary PD (Polymeropoulos et al. 1997). Soon after, the aSyn was identified as the major component of LB (Spillantini et al. 1998). Later on, two other missense mutations were also associated with early-onset, familiar cases of PD, A30P and E46K (Kruger et al. 1998; Zarranz et al. 2004). And more recently, three new missense mutations have been also identified: H50Q, G51D and A53E (Appel-Cresswell et al. 2013; Proukakis et al. 2013; Fares et al. 2014; Lesage et al. 2013; Pasanen et al. 2014). At the same time, the presence of two or three copies of the wild-type aSyn gene were linked to early-onset, familiar cases of PD (Chartier-Harlin et al. 2004; Singleton et al. 2003). Moreover, animal models based on wild-type aSyn overexpression (Masliah et al. 2000; Rockenstein et al. 2002) as well as early onset disease-related mutants A53T and A30P (van der Putten et al. 2000;

Neumann et al. 2002) display a wide range of pathological features and motor symptoms resembling those observed in human patients (Extensively reviewed in Crabtree & Zhang 2012). Accordingly, all these evidences originally pointed to a pivotal role of aSyn in the pathophysiology of PD.

## 2. ALPHA-SYNUCLEIN

aSyn (together with β- and γ-synucleins) belongs to the family of the synucleins, a group of closely related, brain-enriched proteins. This 140 aa-residue protein is largely located in neuronal presynaptic terminals (Kim et al. 2004) and in the nucleus (Yu et al. 2007) of dopaminergic neurons, as well as within astrocytes, microglia and oligodendroglia (Austin et al. 2006; Mori et al. 2002; Richter-Landsberg et al. 2000). In particular, it is found in the neocortex, hippocampus and SN (Kim et al. 2004) and in other brain regions. ASyn belongs to a group of proteins described as natively/intrinsically unfolded proteins (Weinreb et al. 1996), meaning that it does not adopt a well-defined globular structure, but instead a broad ensemble of dynamically interacting and largely disordered conformations. This unfolded nature makes aSyn capable of interacting with a wide range of proteins (indeed more than 50 proteins have been identified to interact with aSyn (Breydo et al. 2012)) as well as with lipid membranes (Jo et al. 2000; Pirc & Ulrih 2015; Lai et al. 2014). Indeed, its promiscuous properties have made difficult the identification of the certain physiological properties. Even though aSyn functions remain to be completely understood, it has been found to play a role in the regulation of the synaptic vesicle pool, acting as an activity-dependent negative regulator of vesicle release (Murphy et al. 2000; Nemani et al. 2010) and, on the other hand, to be a positive regulator of the synaptic transmission by modulating the late steps of exocytosis (Chandra et al. 2005; Burré et al. 2010). Furthermore aSyn regulates brain lipid metabolism (Cabin et al. 2002; Castagnet et al. 2005; Golovko et al. 2009). In addition and

directly related with the dopaminergic system, aSyn has been found to be implicated in the dopamine (DA) metabolism network. First, aSyn interacts and has an inhibitory effect on the enzyme Tyrosine hydroxylase (TH), the rate-limiting enzyme in the DA synthesis chain (Perez et al. 2002). Moreover, aSyn interacts with PP2A (a serine phosphatase), thereby promoting its activity and changing the balance of the TH from the phosphorylated, highly active form to the non-phosphorylated, less active form (Peng et al. 2005). And finally, aSyn can inhibit aromatic amino acid descarboxylase wich definitely converts the inactive metabolite L-DOPA into the active neurotransmitter DA (Tehranian et al. 2006).

Currently, there is considerable accumulated evidence indicating a principal role of aSyn in the etiology of PD, in which the conversion of aSyn from soluble monomers to soluble oligomers and, ultimately, into aggregated amyloid-like insoluble fibrils is a key event in PD pathogenesis (Chiti & Dobson 2006). Indeed the amyloid fibrils, that are the main component of the aforementioned LB and Lewy neurites, share physicochemical properties with those found in AD and other amyloid related diseases e.g. rich β-sheet structure, specific staining, etc. (Reviewed in (Chiti & Dobson 2006)). Firstly the amyloid fibrils were seen as the main neurotoxic molecular species. But accumulating evidences showing the toxicity of aSyn oligomers shifted this perception (Roodveldt et al. 2012; Kalia & Kalia 2015). However, the cellular and molecular mechanisms underlying the pathological actions of aSyn are still not completely understood.

Traditionally, aSyn has been viewed as an exclusively intracellular, cytoplasmic protein that is highly expressed in dopaminergic neuronal cells. However later studies have shown that aSyn is also normally present in extracellular biological fluids, including human CSF(extensively reviewed and analysed in (Gao et al. 2014) and more recently (Zhou et al. 2015)) and blood plasma (Yang et al.

2016; Shi et al. 2014). Shi and colleagues found that aSyn is actually transported from CSF to blood plasma and importantly part of these aSyn is associated with CNS specific exosomes; interestingly they found increased levels of these CNS exosome associated aSyn in plasma from PD patients relative to controls and that these levels correlated with disease severity(Shy et al 2014). Furthermore novel techniques have provided tools to better measure total levels of aSyn in plasma, in order to solve results heterogeneity; in this way, increased total-aSyn and phopho-serine 129-aSyn levels have been found in plasma from PD patients compared to healthy subjects (Yang et al. 2016; Stewart et al. 2015; Wang et al. 2012). Moreover, El-Agnaf and colleagues showed an elevated content of oligomeric aSyn species in plasma from PD patients compared to controls (El-Agnaf et al. 2006). On the other hand, while lower levels of total aSyn than normal have been largely described in CSF from PD patients, other studies show increased levels of oligomeric aSyn and consequently oligomeric aSyn/total aSyn ratio in the CSF from PD (Gao et al. 2014; Zhou et al. 2015). In addition, elevated aSyn staining have been found in several post and ante mortem PD patient tissues, including Gastro intestinal tract, Heart , Salivary gland and Vagus nerve (Beach et al. 2013; Ghebremedhin et al. 2009; Del Tredici et al. 2010; Cersósimo et al. 2011). All these findings seem to indicate that changes in the levels and characteristics of extracellular aSyn are associated with the disease progression and prognosis.

Even though membrane permeability from dying cells was thought to be one contributing source, it was recently demonstrated that the main source of extracellular aSyn are actually healthy intact cells (Ulusoy et al. 2015). Two main mechanisms have been proposed, the principal one thought to be through ER/Golgi independent, non-classical exocytosis (Lee 2008; Jang et al. 2010). However, recent findings point to a second way of secretion of aSyn

associated to vesicles (Emmanouilidou et al. 2011; Kunadt et al. 2015). Moreover, the release of aSyn can be enhanced upon exposure to several cell stressors, such as oxidative stress (Jang et al. 2010; Tyson et al. 2016). On the other hand, extracellular aSyn has been shown to be taken up by neuronal and microglial cells in vitro and in vivo (reviewed in (Tyson et al. 2016). Although the nature of the mechanism involved is still controversial, aSyn has been shown to enter the cells by simple diffusion (Lee et al. 2008) and by active endocytosis (Sung et al. 2001; Hansen et al. 2011; Holmes et al. 2013). In addition, recent studies have provided strong evidence for a neuron-to-neuron and neuron-to-non-neuronal cell transmission of aSyn aggregates and their associated cytotoxicity, in cellular and mouse models of PD (Dehay et al. 2015; George et al. 2013; Vermilyea & Emborg 2015), pointing to a prion-like property of aSyn aggregates (Reviewed in (Tyson et al. 2016; Emmanouilidou & Vekrellis 2016)) and highlighting the importance of extracellular aSyn in the pathogenic mechanism of α-synucleinopathies (Lim et al. 2016).

Biochemical studies have shown that the ensemble of aSyn conformers is perturbed by early-onset related mutations (Lázaro et al. 2014). Conversely, all the familiar PD related mutations do not show the same perturbed characteristics, while E46K, A53T, A30P and H50Q variants show increased oligomer formation rates, A53E and G51D show decreased aggregation rates (Li et al. 2001; Fredenburg et al. 2007; Fares et al. 2014; Ghosh et al. 2014). In addition, A30P shows delayed fibril formation (Li et al. 2001) and, together with A53T, A53E and G51D, impaired membrane binding (Fares et al. 2014; Ghosh et al. 2014). Presumably as a result of the differences in their structural, biophysical and biochemical characteristics, the different variants have been reported to have different cytotoxic effects, and this cytotoxicity to be mediated by different pathways (Reviewed in (Dettmer et al. 2016).

Nevertheless, the factors contributing to both familial and sporadic cases of PD are far from being understood.

## 3. IMMUNE SYSTEM

The Immune system (IS) comprises an ensemble of cells, tissues and organs organised to protect our body from potential harmful agents. One of the key features of the IS is its capacity to discriminate between self and non-self, and even to detect when "self" is also harmful (i.e. cancer). Generally the IS can be differentiated into two sub-systems known as Innate immune system and Adaptive immune system. The Innate IS provides the first line of defence and it is independent from previous contacts with the pathogen; by contrast the Adaptive IS provides a long lasting immunity against pathogens due to its capacity to develop a named 'immune memory'. This capacity allows the system to perform a more efficient response against subsequent exposures to the same antigen. The Innate response could define the profile of the adaptive response while the adaptive response could, after its activation, secrete mediators that will affect the innate system, activating or inhibiting its activity.

The innate IS is mostly built up of myeloid cellular components. In addition, it is characterized by a fast response against pathogens and by a similar response against the same agent upon different exposures. Pathogen recognition is carried out by the identification of the so-called Pathogen Associated Molecular Patterns (PAMPs), which are molecular moieties or structures highly conserved among a wide range of microorganisms. These PAMPs are recognized by receptors known as Pattern Recognition Receptors (PRR), present in all the innate IS common cell types: neutrophils, eosinophils, basophils, macrophages, mastocytes and dendritic cells (Medzhitov & Janeway Jr. 2002; Akira et al. 2006). As a result of the recognition of the PAMPs by the PRR, a cascade of signalling pathway activation ends up with the expression and release of pro-

inflammatory cytokines, chemokines, interferons (IFNs) and co-stimulatory molecules (Medzhitov & Janeway Jr. 2002; Akira et al. 2006), while the exact response profile will depend on the pathogen being recognised. Thus far, three families of PRR have been identified: C-type Lectin Receptors, Nod-like Receptors and Toll-like Receptors (TLRs) (Iwasaki & Medzhitov 2010; Trinchieri & Sher 2007). It is important to mention that the expression of these PRRs it is not exclusive for innate IS cells but has also been detected in several other cell types, including adaptive IS cell types such as B and T cells, which have been found to express TLRs.

TLRs sensing function has demonstrated to be a key point in the initial stages of pathogen identification. TLRs are essential for the correct control and inhibition of the early proliferation of pathogens as well as for recruitment and further activation of necessary elements belonging to adaptive IS. They also play a critical role in the identification of so-called 'Damage Associated Molecular Patterns' (DAMPs) derived from tissue damage, produced either as a result of pathogen invasion, or to be caused by the action of the IS itself or by other processes (i.e. tumour invasion or traumatic injury). TLRs are type1 transmembrane proteins which are evolutionary conserved from insects to vertebrates. To date 13 different TLR gene products in mammals and 11 in humans, have been identified (Akira et al. 2006; Bauer et al. 2009).

As sensors of common pathogenic components, TLRs develop several key functions in the important intersection between innate and adaptive immune systems. For instance, TLRs can induce a signalling cascade that consequently determines the maturation of dendritic cells, secretion of pro-inflammatory cytokines and the further induction and polarization of the adaptive immune response. Except for TLR3, all the TLRs employ the adaptor protein MyD88 to transduce their signalling pathways. After the engagement of the TLRs by their

cognate ligands, MyD88 recruits different proteins that ultimately result in the production of pro-inflammatory cytokines through the induction of the transcription factor κB (NFκB) pathway (Lehnardt 2010).

In addition, TLR family members can be divided into two subgroups depending on their subcellular localization: cell surface receptors (TLR-1, 2, 4, 5 and 6), mainly involved in the recognition of structures present in bacteria or fungi; and intracellular compartment receptors (TLR-3, 7, 8 and 9), primarily involved in the recognition of viral or bacterial nucleic acids. The correct cellular location of TLRs is thought to be of key importance for their function. First of all, the location compartment affects the proper accessibility to antigens while the environment could also determine the recognition; it is known that the interaction between nucleic acids and TLR-3, 7 and 9 depends on the acidic pH only reached inside endolysosomes (Blasius & Beutler 2010). And secondly the correct compartmentalization of the TLRs is thought to be essential for maintenance of self-tolerance, keeping self-antigens inaccessible for TLR recognition (Kawai & Akira 2010). Moreover, the function of TLRs depends on receptor complexes formed with other proteins and this property can determine the exact response provided against each antigen. Moreover, the formation of receptor complexes could explain, at least in part, differences observed between responses elicited by different antigens that bind same TLR and between responses produced by an antigen on different cell types. For instance, TLR4 needs the presence of the co-receptor MD2 to generate its response. However, some cell types also express homologue MD1, and as a result the response against bacterial lipopolysacharide (LPS) could be modulated, generating a non-canonical downstream signalling response (Gorczynski et al. 2006). In the same way, TLR2 can recognise the broadest range of ligands amongst TLRs by forming functional dimers with other TLRs;

specifically TLR1/TLR2 dimers are responsible of recognising triacylated lipopeptides from gram-negative bacteria and mycoplasma, while TLR2/TLR6 dimers recognise diacylated lipopetides from gram-negative bacteria and mycoplasma.

Although it is correct to say that TLRs have evolved to recognise pathogenic, foreign structures, as far as mammals and specifically humans, do not produce flagelin or lipoteichoic acids (Medzhitov & Janeway Jr. 2002), accumulating evidence showing the recognition of endogenous molecules by different TLRs point to another growing theory that holds that, further to its known functions, the Immune System has evolved also to detect and respond to endogenous danger signals (Seong & Matzinger 2004). Nowadays several endogenous proteins and molecules have been identified to bind and be recognised by the TLRs and other innate IS receptors. As we have said, the correct compartmentalization of TLRs is essential for their function, by avoiding the contact between TLRs and possible self-antigens. However, under some situations, e.g. cell necrosis or traumatic tissue damage, the cell content could be released to the extracellular milieu. Under these scenarios, molecules that normally are not able to interact with TLRs become available and could be used as DAMPs by the IS in order to respond to damage. In spite of the fact that activation of the IS in response of these DAMPs could be of benefit, cleaning the damaged tissue and liberating trophic factors and consequently promoting tissue repair, in some cases it could degenerate and become deleterious.

In fact, all the accumulating knowledge about the signalling pathways related to TLRs has provided a growing field of potential therapeutic strategies in order to properly modulate possible pathological responses. (Liu et al. 2010; Takeda & Akira 2004; Romagne 2007; Kato et al. 1994). However, it is also important to remark that the complete inhibition of the response mediated by TLRs

signalling has been related with highly deleterious immune-depressed state (Akira et al. 2006).

**Table TLR ligands** (Jiménez-Dalmaroni et al. 2016)

| Type of TLR | Microbial Ligands (PAMPs) | Potential endogenous Ligands (DAMPs) |
|---|---|---|
| TLR2 **(in association with** TLR 1 **or** TLR6**)** | Lipomannan (Mycobact.) Lipoteichoic acids (Gram-positive bact.) di-acylated and tri-acylated bacterial lipopetides | Hsp60, Hsp70, Hsp90, HMGB-1, Gp96, Biglycan, SP-D |
| TLR4 | LPS (Gram-negative bact.) | Biglycan, Hsp60, Hsp70, Hsp90, Fibrinogen, Fibronectin, Hyaluronic acid, OxLDL (assoc. With **TLR6**), β-Amyloid (assoc with **TLR6**) |
| TLR5 | Flagelin ? | |
| TLR3 | dsRNA (virus) | mRNA (necrotic cells) |
| TLR7 | ssRNA | ssRNA, Imiquimod |
| TLR8 | ssRNA | ssRNA, µRNA |
| TLR9 | CpG motif (Bact. And virus) | Self DNA |

## 3.1 IMMUNE SYSTEM INSIDE THE CENTRAL NERVOUS SYSTEM

For decades, the CNS was considered as an immune privileged organ, primarily due to the presence of the Blood Brain Barrier (BBB). The BBB is a highly selective system constituted by brain endothelial cells connected by tight

junctions and astrocytes that surround the CNS vascular system (Chow & Gu 2015). This structure can limit the presence inside the brain of most of the IS actors, i.e. preventing the pass of Lymphocytes, antibodies and/or complement proteins (Ransohoff & Engelhardt 2012; Daneman & Prat 2015) . As an example, antigen presentation is a key process in the immune response against invading pathogens. But this process was thought to be limited or inexistent inside the CNS due to the presence of the BBB. Firstly, antigen presentation requires the action of the Antigen Presenting Cells (APCs). These cells are capable of phagocytosing and processing micro-organisms and foreign molecules, and present them as antigenic peptides together with the class II major histocompatibility complex (MHC-II), on its cell surface. Different APCs exist most tissues in order to sense the corresponding organs to detect invading pathogens. Upon activation, APCs are able to engage and activate T cells inside local lymphatic draining glands. After its activation, T cells start proliferating and, upon gene rearrangement, they differentiate by increasing its affinity for a concrete antigen. These processes permit further re-stimulations against future re-exposures, the named immune memory. However, the view of the CNS as an Immune privileged system has been widely confronted and reviewed. Indeed a growing body of evidence point to the CNS as a target of several immune disturbances mediated by diverse mechanisms (Lehnardt 2010; Louveau et al. 2015; Guerriero et al. 2016; Vieira et al. 2015; Surendranathan et al. 2015; Wang et al. 2016). To support this statement, there are several observations: first of all, astrocytes, microglia and endothelial cells, all of them CNS constituent cells, have been found to be able to express MHC-II molecules on their cell surface and to be recognised by $CD4^+$ T cells, while neurons are able to express MHC-I, recognizable by $CD8^+$ T cells. Secondly, we now know that microglia and astrocytes can act as APCs in the CNS (Gimsa et al. 2013; Michell-Robinson et al. 2015). And finally, the existence of lymphatic drainage from CNS

to cervical lymph nodes (Weller et al. 2010) and more recently the presence of lymphatic vessels on the surface of the human brain has been demonstrated (Louveau et al. 2015; Aspelund et al. 2015). As a consequence, the actual degree of sealing of the BBB is currently under much debate (Su & Federoff 2014; Heneka et al. 2015).

As aforementioned, the BBB surrounds all CNS vascular systems. However, some areas of the CNS are considered BBB-free areas, such as the choroid plexus blood-CSF barrier and the outer CFS-brain barrier, the Pia Arachnoid. Moreover, under neural inflammatory insult the tight junctions of the BBB are known to suffer a rearrangement that permits peripheral lymphocytes to cross the BBB entering the brain (Stolp et al. 2013), again in direct opposition to the immune-privilege status of the CNS. In addition, the BBB has been demonstrated to show in its surface different receptors and transporters that enable the capacity to sense the environment. Consequently, the BBB is without any doubt essential not only for the maintenance of homeostasis inside the brain parenchyma, but also as a key ruler in neuro-inflammatory processes, regulating the proper entry of IS mediators inside the CNS, such as lymphocytes, cytokines, chemokines and/or antibodies (Daneman & Prat 2015; Chow & Gu 2015).

Under pathological conditions, increased amount of immune cells, e.g. B and T Lymphocytes, can be found inside the meninges and the CSF. As a result, these cells can access the brain parenchyma, crossing the BBB. As aforementioned, the BBB regulates the proper transit of molecules and cells throughout cerebral vascular system, and this function is essential for the maintenance of the cerebral homeostasis. Actually, the dysregulation of the BBB traffic has been associated with certain pathologies. One example is multiple sclerosis, in which the abnormal infiltration of IS cells into the CNS has been identified as a

common characteristic in a large number of patients (Antel & Owens 1999; Adams et al. 1989; Prineas & Wright 1978). Taken together, these observations point to the importance of studying the mechanisms that control the migration of cells, and particularly IS mediator cells, throughout the BBB (Daneman & Prat 2015; Su & Federoff 2014; Heneka et al. 2015).

As for the rest of our system, in the Nervous System, cytokines are part of the machinery responsible of the correct activation, proliferation, differentiation and they ultimately define the phenotype of different cell types. Moreover, they participate in inflammatory response, tissue remodelling and repair mechanisms. Importantly, two essential features of cytokines are their pleiotropism, which means that one cytokine can display different effects in different cell types or different scenarios; and their redundancy, meaning that different cytokines can elicit the same response. Inside the CNS, astrocytes and microglia cells are the principal sources of cytokines(Hanisch 2002; Dong & Benveniste 2001). Although other cells have been documented to produce cytokines under certain circumstances, for example oligodendrocytes and Schwann cells (Zeis et al. 2015; Scheib & Höke 2016)

Cytokines are involved in complex signalling networks between Nervous System and IS. Accordingly, cytokines secreted by astrocytes or microglial cells are capable of spreading the inflammatory response, for example recruiting IS mediator cells such as lymphocytes. On the other hand, the IS has tools to self-control the response in order to limit and control the inflammatory response after accomplishing its function. That is, for instance, the existence of cytokines with inhibitory or regulatory functions; e.g. IL-10. However, when these self-limiting mechanisms fail, or turn out dysregulated, the whole system could fall into a pathological state. Certainly, the suppression or the spread of the inflammatory response is under the control of several factors: a) the actual

activation state of the cells, defined by their expression of different factors such as cytokine receptors, both pro and anti-inflammatory, which determine the effective susceptibility to stimulation; b) the concentration and exact localization of cytokines, both pro and anti-inflammatory; c) the temporal sequence of the exposition to these cytokines by each group of effector cells. Therefore, the interaction of all these complex factors will result in the ultimate response generated.

As mentioned earlier, microglia cells are the resident macrophages of the CNS. So they represent the first line of defence against foreign agents. As components of the innate IS they express large numbers of PPRs, and specifically TLRs, to recognise possible foreign agents (Lehnardt 2010; Kawai & Akira 2010). Furthermore, the TLR system has been related with either neuroprotective processes or neurodegenerative diseases, highlighting the crucial importance of completely understanding this system(Jiménez-Dalmaroni et al. 2016; Kawai & Akira 2010; Alvarado & Lathia 2016).

Under non-pathologic insult, microglia rest quiescent with a characteristic morphology with long and ramified processes. However, microglia can become active under several conditions leading to a neuro-inflammatory scenario: Nervous System infections, neural injury and neurodegenerative diseases among others (Jin & Yamashita 2016; Hu et al. 2015). Upon activation, microglial cells change their morphology to an amoeboid state characterised by enlarged soma with less and sorter ramifications. In addition they acquire phagocytic capacity and start producing and secreting numerous cytokines and chemokines. Moreover, after activation microglia cells increase the surface expression of important receptors like MHC-II involved in antigen presentation function (Aloisi 2001; Lehnardt 2010; Glass et al. 2010; Jin & Yamashita 2016).Indeed, due to the importance of active microglia and its deleterious

activities, inside the nervous system there are different overlapping ways operating to fine control this process under physiological conditions.

As has been noted, microglia cells main function is to sense the neuronal tissue to detect damage or foreign agent's invasion, but they also show an important restorative capacity. As a consequence of all these processes, microglia is capable of eliminating parenchymal residues and promoting neuronal healing (Lehnardt 2010; Aloisi 2001; Jin & Yamashita 2016). Conversely, under certain neurodegenerative pathologies, the control network fails, resulting in neuronal damage. In addition, the same mediators have been related with both processes. As explained earlier, pro-inflammatory mediators such as TNF-α, IL-1, IL-6, IL-12, IL-18; chemokines like IL-8, MIP, MCP o RANTES; and even immune modulatory cytokines like IL-10 or TGF-β mediate in the inflammatory process under physiological conditions. By contrast, different processes, most of all currently unknown, can lead to a dysregulation of the normal response networks and can be related with neurodegenerative diseases (Jin & Yamashita 2016; Heneka et al. 2015; Hu et al. 2015).

Recent findings have demonstrated the importance of the IS and the inflammatory response, as a factor that can promote, and even expand, neuronal damage associated with CNS chronic diseases. For example the role of the IS in the progression of AD (Richards et al. 2016; Heneka et al. 2015; Brück et al. 2016), and importantly PD that will be discussed later. In addition this relation has been reported in acute diseases, like cerebral stroke (Kaur & Ling 2008). In fact, some of best known neurological diseases, such as ALS or MS, share the implication of IS activation as a primary ruler on the development of the disease (Naegele & Martin 2014; Evans et al. 2013).

Although we have been speaking about active microglia, nowadays we know that the activation of monocyte/macrophages covers a wide range of different polarized states. On the one hand, we have the so-called M1 activation state. Firstly described in 1962 by Mackaness, the M1 or classical activation state is the one normally associated with inflammation and pro-killing activities(Mackaness 1962). The M1 state is characterized by its secretion of cytokines, chemokines and surface marker expression profile. Commonly, M1 macrophages secrete pro-inflammatory cytokines such as TNF-α, IL-6, IL-12 and IL-1β, and chemokines, such as CCL2 and CXCL10. They also express the active-related markers MHC-II and CD86 on the surface, while they increase the expression of the inducible pro-inflammatory-related form of the nitric oxide synthase enzyme iNOS (Nau et al. 2002; Martinez et al. 2006). As expected, to induce this 'classical active' state the paradigmatic stimuli are IFN-γ and the activation though the TLRs family by their known cognate agonists, e.g. LPS recognition by TLR-4 (Hu & Ivashkiv 2009). On the other hand, we have the activation state generally associated with immune modulation and healing. As opposed to the more specific M1 state, the M2 phenotype, also called 'alternative activation' state, has been related with a much broader range of profiles, and therefore it has been divided into three subtypes. The first one described was the M2a state, primary related to host defence against parasites and allergy. Its paradigmatic stimulus is the Th2 emblematic cytokine IL-4. The M2a state is characterized by the expression of IL-10 (common to all M2 states) and polyamines, the surface expression of CD206 and importantly used as a marker, the cytosolic expression of Arginase 1 (Arg1). Secondly, related to upregulated phagocytosis and immune modulation, we have M2b state. Commonly induced by the activation of the TLR system followed by IgG constant domain, the M2b is characterised by large secretion of IL-10 and low to moderate amounts of pro-inflammatory cytokines like TNF-α, IL-6 and IL-1β,

accompanied by the surface expression of CD86 (Sánchez-Mejorada & Rosales 1998; Takai 2005; Edwards et al. 2006). And finally we have the M2c state, most related with tissue repair and deactivation of M1/Th1 immune responses (Fiorentino et al. 1989; Glocker et al. 2009). The most paradigmatic inducer of this state is the immune-modulatory cytokine IL-10, interestingly secreted by all the other two states. The activation of the M2c state is accompanied by the secretion of IL-10, CXCL13, CXCL4 and matrix proteins (Park-Min et al. 2005). In conclusion M2 in opposition to M1 lead to a broader range of responses while all of them are related with immune modulatory actions. Despite the fact that microglia has a different embryonic origin than monocyte/macrophages, the M1/M2 paradigm has been proposed to be similar in microglia and macrophages (Durafourt et al. 2012), introducing an interesting field of research that is currently under focus (Extensively reviewed in (Moehle & West 2014; Hu et al. 2015)).

## 3.2 NEUROINFLAMMATION IN PARKINSON'S DISEASE

As we have largely mentioned above, the active role of the IS in most neurodegenerative diseases has been extensively shown. Specifically, in the last few years, it has become evident that the immunological component is of central importance in PD pathogenesis and progression. Certainly, the presence of a robust inflammatory response mediated by activated microglia and reactive astrocytes in affected areas of the SNpc is an important pathological feature in PD brains (Brück et al. 2016). Indeed, inflammation in the CNS and sustained over-activation of microglia, i.e. reactive microgliosis, are currently believed to be actively involved in the pathogenesis of various neurodegenerative diseases including PD, AD, MS, ALS (Gao & Hong 2008; Glass et al. 2010; Kim & Joh 2006; Long-Smith et al. 2009; Heneka et al. 2015; Richards et al. 2016). During the past 10-15 years, whether microglial activation

ultimately protects or actually exacerbates neuronal loss in the context of PD and other related diseases had been under strong debate (Delgado & Ganea 2003; Gao & Hong 2008; Halliday & Stevens 2011; Sánchez-Pernaute et al. 2004; Vila et al. 2001; Wu et al. 2002; Wyss-Coray & Mucke 2002; Wu et al. 2014; Chen et al. 2016). Evidence of microglial attack and neuroinflammation in PD is supported by findings from epidemiological studies, animal models, and cell culture experiments (Allen Reish & Standaert 2015; Kannarkat et al. 2013). The first evidence of microglial implication in PD was the finding of active microglial cells surrounding degenerating dopaminergic neurons in SNpc of PD patient's post-mortem brains (McGeer et al. 1988). Since in vivo PET imaging has confirmed the presence of active microglia in active degenerating sites and furthermore, this presence directly correlates with PD severity both clinically and pathologically (Ouchi et al. 2005). In the same way, several molecular inflammatory mediators such as cytokines and chemokines have been found to be upregulated in post-mortem brains and in CSF and blood serum of PD patients. Specifically, IL-1β, IL-6, TNF-α and IFN-γ are increased in brains and fluids from PD patients compared to controls (Brodacki et al. 2008; Blum-Degen et al. 1995; Mogi, Harada, Riederer, et al. 1994; Mogi, Harada, Kondo, et al. 1994). Importantly Kuziorowsky and co-workers found a direct correlation between IL-6 levels and disease severity (Koziorowski et al. 2012)(Kuziorowsky et al 2012) while dopaminergic neurons have shown to be more sensitive to proinflammatory cytokines like IFN-γ and TNF-α (Tansey & Goldberg 2010). In addition, epidemiological studies have revealed that taking ibuprofen anti-inflammatory agent regularly is associated with lower risk of developing PD (X. Gao et al. 2011; Samii et al. 2009). Taken together, all these findings support the concept that inflammatory attack is contributing to dopaminergic neuronal loss. Although currently it is not stablished whether the implication of the IS is etiologic or a resultant process in the pathophysiology of the PD, it is important

to note that the activation of the IS has been found to occur prior to neuronal loss in different animal models (Chung et al. 2009).

At the moment, two major groups of PD animal models are available: Toxin-based models like MPTP, 6-OHDA or Rotenone, and Transgenic animal models, which are based on the over-expression of aSyn. In both groups, microglia has been found to be in activated state (McGeer et al. 2003; Akiyama & McGeer 1989; Sherer et al. 2003; Theodore et al. 2008; Harms et al. 2013; Chung et al. 2009; Watson et al. 2012), while the suppression of the inflammatory response, either by anti-inflammatory drugs or by inhibiting key proinflammatory mediators, is capable of ameliorating and even completely inhibiting neuronal loss (Du et al. 2001; Harms et al. 2011; Tanaka et al. 2013). Moreover, it has been recently reported that treatment with CSF from PD patients strongly affects cultured microglial cells, resulting in reduced cell growth, morphological changes, as well as increased content and aggregation of aSyn (Schiess et al. 2010). This illustrates how microglia itself, and not only dopaminergic neurons, can be highly affected by the medium in a PD scenario. The current picture is that microglial-mediated inflammation could have a dual effect, as seems to happen in other neurological pathological processes such as MS or Traumatic Brain Injury (Moehle & West 2014; Jin & Yamashita 2016). In this scenario microglia activation can be thought of being neuroprotective during the initial stages and deleterious/neurodegenerative at the mid and advanced stages of disease.

## 3.3 ALPHA-SYNUCLEIN AND INNATE INMUNE SYSTEM IN PARKINSON'S DISEASE

The results gathered thus far using the different PD animal models have substantially increased our understanding of PD's pathogenesis by usually providing different but probably complementary information. As

aforementioned, while the MPTP mouse model of PD indicates that inflammation in the SN can be self-propagating and leads to progressive neurodegeneration, the aSyn transgenic animal model demonstrates that overexpression of this endogenous protein can certainly provide a powerful source of inflammation (Allen Reish & Standaert 2015). Whether microglial activation is essentially caused by the release of aberrant aSyn species to the extracellular space, (Reynolds, Glanzer, et al. 2008; Wersinger & Sidhu 2006; Zhang et al. 2005; Halliday & Stevens 2011; Harms et al. 2013; Roodveldt et al. 2010; Brück et al. 2016) or otherwise, that neuronal dysfunction and/or death itself drives microglial immune responses in an aSyn-independent manner (Mandel et al. 2005; McNaught et al. 2010; Walker et al. 2016; Leak 2014), is still under debate. However, there is ample accumulated evidence pointing at aSyn as the main trigger of microglial activation in PD (Roodveldt et al. 2008; Ren et al. 2016). For example, several studies have demonstrated that extracellular and nigral aSyn-containing aggregates are often surrounded by activated microglia or inflammatory mediators in PD brains (McGeer et al. 1988; Yamada et al. 1992; Chao et al. 2014), similarly to what has been described for amyloid plaques in AD (Griffin et al. 2006). Moreover, the extent of microglial activation in the SN from PD patients has been found to be correlated with the degree of aSyn accumulation (Croisier et al. 2005) and with increased aSyn levels as evidenced by in vitro (Kim et al. 2009; Klegeris et al. 2008) and in vivo (J.-K. Lee et al. 2009; Sanchez-Guajardo et al. 2010) studies, strongly supporting the view that the protein has a major role in phenotypic changes of microglia (Reviewed in (Brück et al. 2016; Ren et al. 2016)). Up to this point, a considerable number of in vivo studies with animal models of PD that directly link aSyn with microglial activation have been reported. It has been demonstrated in mice that overexpression of aSyn alone (by using adeno-associated virus, AAV) is sufficient to trigger neuroinflammation, involving not

only classical microglial activation but also stimulation of adaptive immunity, preceding the appearance of overt neurodegeneration signs (Theodore et al. 2008; Su et al. 2008; Su et al. 2009; Halliday & Stevens 2011). In line with this finding, different rat and mouse models overexpressing Wt or mutant variants of aSyn variant in the SNpc revealed dramatic changes in cytoskeletal protein levels and activated microglia-mediated neuroinflammation in the striatum (with increased release levels of IL-1β, IFN-γ, and TNF-α proinflammatory cytokines), well before neuronal loss was evident (Su et al. 2008; Su et al. 2009; Chung et al. 2009; Chesselet et al. 2012). Importantly, another study using a AAV rat PD model showed that overexpression of Wt aSyn in the SN not only leads to persistent microglia activation, but that depending on the degree of aSyn-induced neurophatology that models either the onset or the late stages of the disease, different microglial responses will occur: upon lower aSyn expression levels where only neurodegeneration occurs, microglia with antigen-presenting capabilities predominate, whereas levels that can induce neuronal cell death correlate with long-term induction of macrophagic microglia (Sanchez-Guajardo et al. 2010), suggesting that microglia may play different roles during disease progression (Sanchez-Guajardo et al. 2010).

The link between neuroinflammation and aSyn dysfunction has been stablished using different approaches. The use of LPS injection in rat (Choi et al. 2010; Sharma & Nehru 2015) or mice (H. M. Gao et al. 2011), to trigger systemic and brain inflammation was used firstly. In the first study, the authors observed increased microglia activation and secretion of proinflammatory cytokines as well as greater nitration of proteins including aSyn, in elderly rats, suggesting that an exaggerated neuroinflammatory response that occurs naturally with aging might contribute to aSyn aggregation and dopaminergic neurodegeneration in PD (Choi et al. 2010). In the second study, the authors

evaluated dopaminergic neurodegeneration, aSyn pathology and neuroinflammation in Wt and transgenic A53T aSyn-overexpressing mice (H. M. Gao et al. 2011). They observed that, while both models initially displayed acute neuroinflammation, only the latter developed persistent neuroinflammation together with chronic progressive degeneration of nigrostriatal dopamine pathway, accumulation of aggregated, nitrated aSyn, and formation of LB (H. M. Gao et al. 2011), suggesting that genetic factors and environmental exposures act synergistically to precipitate the development of PD. In addition recent studies have found a link between stress and neuroinflammation with further aSyn dysfunction leading to dopaminergic cell death and providing another prove of the central role of the microglia and its neuroinflammatory capabilities in PD (de Pablos et al. 2014; Sugama et al. 2016).

On the other hand, microglial cells from aSyn-knockout mice have been shown to exhibit a remarkably different morphology compared to Wt cells (Austin et al. 2006), displaying elevated levels of secreted pro-inflammatory cytokines such as TNF-α and IL-6 after activation, indicating that aSyn plays a critical role in modulating the microglial activation state.

In the last few years, several in vitro studies have focused on the effects of extracellular aSyn on microglial activation. Zhang et al. (Zhang et al. 2005) first reported that exogeneous, aggregated aSyn cause activation of microglial cells, which then become toxic towards cultured dopaminergic neurons. Their results indicate that microglial phagocytosis of aSyn and activation of NADPH oxidase, are critical in aSyn-induced microglial activation and neurotoxicity. This finding is highly relevant considering that, as has been mentioned, aSyn has been shown to be present in body fluids and brain parenchyma of health and disease individuals and moreover to be taken up by neuronal and microglial cells in

vitro and in vivo (Tyson et al. 2016; Emmanouilidou & Vekrellis 2016; Lim et al. 2016).

Up to this point, research on aSyn-mediated cell response has focused primarily on the effects of aSyn on neuroinflammation (Benner et al. 2008) or microglial activation (Cookson 2009; Reynolds, Kadiu, et al. 2008; Thomas et al. 2007; Zhang et al. 2007; Zhang et al. 2005; Harms et al. 2013; Kim et al. 2016) with aSyn in its aggregated form. Interestingly, Reynolds and coworkers (Reynolds, Glanzer, et al. 2008) have found that nitrated, aggregated aSyn (N-aSyn) has a stronger stimulating effect on microglia than that of nitrated but non-aggregated aSyn. In addition, several investigations have found that N-aSyn, which has been detected in LB of human brains with PD (Giasson et al. 2000) and has been linked to neurodegeneration in PD mouse models (Benner et al. 2008; Gao et al. 2008), induces a neurotoxic inflammatory microglial phenotype that accelerates dopaminergic neuronal loss (Biasini et al. 2004; Thomas et al. 2007; Zhang et al. 2005; Zhou et al. 2005). By integrating genomic and proteomic techniques, Gendelman and colleagues created a fingerprint of microglial cell activation following its interactions with aggregated N-aSyn in cell culture (Reynolds, Glanzer, et al. 2008), indicating that the activation, which was found to be capable of mediating dopaminergic neurotoxicity, is mainly mediated by the NFκB pathway (Reynolds, Glanzer, et al. 2008). However, whether extracellular aSyn contains the same modifications than the protein found in LB (Anderson et al. 2006; Giasson et al. 2000; Hodara et al. 2004), which is a typically pro-oxidative environment, is still uncertain (Lee 2008).

Over the last few years, certain differential consequences of the presence of non-aggregated aSyn in the extracellular medium in glia have been reported. It has been observed that, in contrast to the aggregated form, monomeric aSyn enhances microglial phagocytosis (Park et al. 2008). A few investigations that

explore the effects of non-aggregated aSyn on the cytokine release profile of potentially relevant cells have been done using monocytic (Klegeris et al. 2008) or macrophage (S. Lee et al. 2009) cell lines, and primary astrocyte (Klegeris et al. 2006) or microglial (Roodveldt et al. 2010; Su et al. 2009; Su et al. 2008) cultures. Indeed, we have observed a strong innate immune response in primary glial and microglial cell cultures elicited by exogenous, non-aggregated aSyn (Roodveldt et al. 2010). Interestingly, a comparative study using unmodified aSyn has recently shown that exogenous non-aggregated aSyn induces higher TNF-α, IL-1β and ROS release levels than aggregated aSyn in microglia (Lee et al. 2010). These and other recent findings point at the importance of exploring the effects on the immune response of aggregated as well as non-aggregated aSyn.

Even though a study using monocytic THP-1 cell line (Klegeris et al. 2008) had shown modest increases in IL-1β and TNF-α secretion levels after stimulation with A30P, E46K, A53T or aSyn variants compared to the Wt protein, there is a lack of a comprehensive study of the effect exerted by non-aggregated aSyn, performed with primary cell cultures.

With this in mind, we analysed the cytokine release profile of primary microglial cultures —which represents a more comparable physiological environment— after stimulation with Wt or the PD-linked aSyn mutants (Roodveldt et al. 2010). Indeed, we found remarkable differences between the aSyn variants in the interleukin and chemokine release profiles and significant effects on the microglial phagocytic capacity (Roodveldt et al. 2010). In particular, we observed marked differences in IL-6 and IL-1β pro-inflammatory cytokines, IL-10 immunoregulatory cytokine, as well as IP-10/CXCL10, RANTES/CCL5, MCP-1/CCL2 and MIP-1α/CCL3 chemokines release levels. Our results indicate that extracellular, non-aggregated Wt aSyn produces a moderate to low pro-

inflammatory response in glia, together with a reduction of the immunoregulatory response, and a moderate stimulation of Th1 chemokine secretion. The A30P and E46K pathological variants, on the other hand, can induce strong pro-inflammatory and immunoregulatory responses, together with marked increases in chemokine release levels. This exacerbated innate immune response might explain the earlier onset and more rapid evolution of these two genetic cases of PD as compared to the sporadic kind. Intriguingly, our results from the pathologically-linked A53T variant showed not to provoke a significant innate immune response, which might suggest that other neurodegeneration mechanisms contributing to the pathogenesis of PD, probably involving the adaptive immune response might exist in this case. Combined with the effect on microglial phagocytosis, our results indicate that these aSyn-induced phenotypes might reflect either a classical (A30P and E36K) or an alternative (A53T) microglial activation state, or a hybrid phenotype (Wt), which could probably explain the different disease progression modes that can occur in PD. Although nowadays all the evidence points to a classical activation state (M1) as the main microglia phenotype contributing to PD progression, it is important to say that microglia, and macrophages, use to display a continuum of phenotypes from M1 to M2 (Martinez & Gordon 2014; Moehle & West 2014). Examples of the presence of these mixture of M1, M2 phenotypes has been largerly described in the prototypical neuro-inflammatory disorder MS (Zhang et al. 2011; Moreno et al. 2014). A similar scenario could be operating in the context of PD, but further investigations will be needed

As aforementioned, upon activation microglia and astrocytes start secreting inflammatory cytokines in order to communicate wither other cells and mount the immune response to counteract disease or injury. The cytokines TNF-α, IL-1β, IL-2, IL-4, IL-6, TGF-α, TGF-β1, TGF-β2 have all been reported to be

increased in the nigrostriatal region and CSF of patients with PD or DLB (Croisier & Graeber 2006; Allen Reish & Standaert 2015). More recently, Williams-Gray and co-workers pointed to TNF-α, IL-1β, IL-2 and IL10 as possible PD biomarkers as far as they found increased levels of all these cytokines in PD patient serum compared to age matched controls (Williams-Gray et al. 2016). In the same way, Kuziorowsky and colleagues found a direct correlation between serum IL-6 and severity of PD (Koziorowski et al. 2012). As a result of aSyn-induced activation of microglia in vitro, a few cytokines and metabolites have been shown to be significantly up-regulated (reviewed in (Roodveldt et al. 2008; Brück et al. 2016): IL-6, IL-1β, ICAM-1, TNF-α, IFN-γ, MCP-1, O2-, iROS, and PEG2, glutamate, and iCys. In general, disease-linked aSyn variants show a stronger effect on cytokines release than does the Wt protein. Interestingly, analysis of the microglia transcriptome by Gendelman and coworkers (Reynolds, Glanzer, et al. 2008) after stimulation with aggregated N-aSyn, revealed a significant up-regulation of the TLR-2 gene. As previously mentioned, TLRs sense the molecular signatures of microbial pathogens, and play a fundamental role in innate immune responses, inducing the expression of diverse inflammatory genes (Kawai & Akira 2010). In the same way, recent findings are pointing to a possible role of the TLR system in the aetiology of PD; TLR2 has been shown to be the link between neuron secreted oligomeric aSyn, microglial activation and the subsequent dopaminergic neuronal damage (Kim et al. 2016). At the same time TLR4 has been related with the recognition of monomeric aSyn and to play a role on its correct clearance (Fellner et al. 2013).Therefore, it seems plausible that cells challenged with aSyn, or with certain forms of aSyn, could become hyper-responsive to inflammatory signals due to changes in its cellular receptors expression or function.

Activated microglia can also produce substantial amounts of superoxide radicals, which may be the major source of the oxidative stress thought to be largely responsible for dopaminergic cell death in PD (Blesa et al. 2015). The generation of ROS by microglia activated by aSyn (Alvarez-Erviti et al. 2011; Wang et al. 2016) can result in oxidation and nitration of proteins, DNA modifications, and lipid peroxidation, leading to neurotoxicity (Zhang et al. 2005; Kim et al. 2015). Oxidation (Ko et al. 2000; Chavarría & Souza 2013) and nitration (Giasson et al. 2000; Chavarría & Souza 2013) of aSyn which in turn can lead to the formation of more aggregates and result in increased cytotoxic effects (Goodwin et al. 2013).

## 3.4 ADAPTIVE IMMUNE SYSTEM IN PARKINSON'S DISEASE

The Adaptive IS, unlike the Innate IS is characterised by the capability of developing specific responses to specific antigens (proteins, pathogens, self-antigens, etc.) and to provide a long lasting immunity against these antigens due to its capacity to develop a so-called 'immune memory'. The General mechanism of action of the Adaptive immunity requires the antigens to be recognised by T cells, usually with the intervention of APCs, and the subsequent activation of B cells to differentiate and produce specific antibodies against such antigens (Fakhoury 2016). In recent years accumulating evidences has pointed at the importance of the Adaptive IS in the initiation and progression of several neuro-degenerative diseases such as AD, ALS and specifically in PD (Sanchez-Guajardo, Barnum, et al. 2013; Allen Reish & Standaert 2015; Fakhoury 2016).

The initial observation in PD patients that a small amount of CD8+ Tlymphocytes occur in proximity to degenerating nigral neurons (McGeer et al. 1988), and the occurrence in LB of components of the classical or antibody-

triggered complement cascade (Yamada et al. 1992) suggested a possible involvement of the adaptive immunity in the PD process. Since then, the relation of the AIS with the pathogenesis of PD has been established from a growing body of convergent evidences. First of all, different works have shown increased numbers of T cells, both CD4+ and CD8+, in SNpc in post-mortem brains of PD patients (Brochard et al. 2009; Farkas et al. 2000). Indeed Brochard and colleages found 10-fold greater infiltration of T cells in PD brains compared with age-matched controls. Additionally, the peripheral IS cell pool has been found to be altered during PD progression. In particular, a reduction of total circulating CD4+ T cells has been observed, while the total numbers of CD8+ T cells seem to remain unaffected (Calopa et al. 2010; Baba et al. 2005; Niwa et al. 2012; Stevens et al. 2012). Importantly, has been found that the existing CD4+ T cells exhibit an increased Th1/Th2 ratio that positively correlates with PD severity (Chen et al. 2015; Baba et al. 2005). While Saunders and co-workers have found increased numbers of effector T cells together with impaired function of regulatory T cells in peripheral blood of PD patients and a positive correlation with their UPDRSIII (Unified Parkinson's Disease Rating Scale Part III) performance (Saunders et al. 2012). Taking together these results point to a pro-inflammatory Th1 scenario during PD progression. In agreement with these findings, different animal models have been found to exhibit similar lymphocytes recruitment to brain parenchyma. Indeed, a remarkable T- and B-cell infiltration into the SN linked to aSyn overexpression was observed at the early stages of disease development, i.e. before the appearance of significant dopaminergic neuronal loss, in a rAAV-aSyn mouse model overexpressing aSyn, reaching levels in the SN of up to 10-fold and 5-fold compared to controls (Theodore et al. 2008). While in an MPTP mouse model the composition of the infiltrating populations was reported to change during disease progression, with increased numbers of CD4+ T helper cells infiltrating during first stages

and changing to increased CD8+ cytotoxic T cells after the microgliosis peak had occurred and significant neuronal loss had been detected(Sanchez-Guajardo et al. 2010; Brochard et al. 2009).

Thus far, accumulated data demonstrate that in the MPTP model of PD, misfolded and aggregated aSyn are secreted from neurons, which promotes pro-inflammatory M1-type microglia and cytotoxic T-cells, therefore amplifying neuronal damage. In sporadic human PD, it is currently unkown which factor triggers disease onset, but it has been proposed that under certain circumstances, a similar set of temporal and mechanistic events could transform neuroprotective microglia and T cells into cytotoxic cells, thereby accelerating disease progression (Appel et al. 2010). This way, activated microglia and the cytokine milieu that they generate might promote T-cell differentiation into different cell subsets in the context of PD (Appel et al. 2010). Indeed, it has been shown that M1 (pro-inflammatory) cells promote, whereas M2 (non-inflammatory) cells reduce, CD4+ Th1 cell proliferation and function (Verreck et al. 2004), but also that, conversely, T-cells can dictate microglial pro- or anti-inflammatory phenotypes (Giuliani et al. 2003; Kebir et al. 2007; Mount et al. 2007)

Whether microglia dictate the specific T-cell phenotype or otherwise, that T-cells dictate the specific microglial phenotype (i.e. M1 vs. M2), is still unknown (Appel et al. 2010). But overall, the communication established between microglia, T cells and neurons seem to indicate that the immune response is not only a consequence of injury, but that it actively contributes to the balance between neuroprotection and neurotoxicity (Appel et al. 2010; Stone et al. 2009).

On the other hand, although infiltration of B cells has not been observed yet into PD patients' brains, the humoral immunity has been implicated in the pathophysiology by several findings. To analyse this possibility, Orr et al. (Orr et al. 2005) analysed the association between nigral degeneration and humoral immune markers in brain tissue of patients with idiopathic or genetic PD and controls. All the patients with PD revealed IgG, but not IgM, binding on dopamine neurons. Moreover, the proportion of IgG-immunopositive neurons showed a negative correlation with the degree of cell loss in the SNpc, and positive correlation with the number of activated microglia. IgG was found to be concentrated at the cell surface of neurons, but also on their LB, and was shown to co-localize with aSyn. These results, in combination with their finding that activated microglia express high-affinity IgG receptors (FcγRI) in both idiopathic and genetic forms of PD, might suggest that the activation of microglia may be induced by neuronal IgG (Orr et al. 2005). The question regarding the functional importance of antibodies against antigen-specific, disease-associated neuronal proteins still needs to be addressed. It has been demonstrated that an IgG fraction purified from serum of PD patients causes death of dopaminergic neurons in vivo following stereotaxic injection in the SN of experimental animals (Chen et al. 1998; He et al. 2002), and the presence of immunoglobulins in PD brain tissue have been proposed to lead to the targeting of dopaminergic nigral neurons for destruction (Orr et al. 2005). Currently, it remains unknown whether these anti-aSyn AAb are neurotoxic, or on the contrary, they actually have a neuroprotective role, as has been shown in a human aSyn transgenic mouse model of PD (Masliah et al. 2005). A recent study has assessed the presence of auto-antibodies (AAb) against all three synucleins in the peripheral blood serum of PD patients and healthy controls (Papachroni et al. 2007; Han et al. 2012). While the presence of AAb against β- and γ-Syn showed no association with PD, multi-epitopic AAb against aSyn were

detected in 65% of all patients, with a strong correlation with the inherited mode of the disease. In addition, a recent study based on measuring AAb levels against monomeric, oligomeric, and fibrillar aSyn in serum from PD patients (Gruden et al. 2011), showed that all three AAb specificities reached the highest values after 5-year of disease duration, and subsided in 10-year sufferers. Intriguingly, there was a ca. 15-fold increase in AAb titre values relative to monomeric aSyn (72% of patients), and a ca. 4-fold increase for aSyn oligomers (56% of patients). Moreover, the authors also found a decline in CD3+, CD4+ and CD8+ T-lymphocyte and B-lymphocyte subsets. Based on these results, they suggest that aSyn toxicity elimination by AAb in early PD pathology might be linked with the decline of lymphocyte subsets reflecting the influence of inflammatory and oxidative stress processes (Gruden et al. 2011)

## 4. IMMUNOTHERAPY IN PARKINSON'S DISEASE

As we have largely explained in previous sections, the relation between disturbances in the IS have been related with disease onset and progression of PD and other neurodegenerative diseases. In this way, different anti-inflammatory compounds have been tested for their capacity to act as therapeutic options, in PD mouse models (Olson & Gendelman 2016). Indeed several epidemiological studies and mouse model experiments, have shown a possible protective effect of taking ibuprofen in PD (Rees et al. 2011). Along the same line, the use of minocycline has been tested due to its higher anti-inflammatory activity, but no effect was demonstrated (NINDS NET-PD Investigators 2006; NINDS NET-PD Investigators 2008). Moreover, different natural compounds, such as Resveratrol or Silymarin, have shown promising results in rodent PD models as treatment agents, because of their anti-inflammatory and antioxidant properties (Haddadi et al. 2014; Lofrumento et

al. 2014). However, anti-inflammatory agents have failed to show clinical utility and further investigations will be required(Olson & Gendelman 2016).

**Table 2. aSyn Vaccination strategies** (adapted from (Schneeberger et al. 2016))

| Vaccination | Mouse Model | Results | Ref. |
|---|---|---|---|
| **Full-length human aSyn** | Mouse,PDGF-a-Syn, D-line | Induction of aSyn–specific IgG Abs<br>Reduction of misfolded aSyn in neuronal cell bodies and synapses<br>Neuropathological improvement correlation with the strength of the immune response | (Masliah et al. 2005) |
| **Full-length human aSyn, Nitrotyrosine modified and unmodified** | Mouse,MPTP-C57BL/6 | Immune responses exacerbate neuroinflammation and nigrostriatal degeneration<br>Activation of peripheral leukocytes<br>Exacerbations are mostly mediated by aSyn–induced/specific Tcells<br>Treg cells attenuated microglial inflammatory responses and led to robust nigrostriatal protection | (Benner et al. 2008; Reynolds, Glanzer, et al. 2008) |
| **Full-length human aSyn** | Rat,rAAV2/5-a-Syn overexpression | High- anti–aSyn antibody response<br>Reduction in PD-typical aggregates;<br>Accumulation of CD4-+, MHC II+ ramified microglia in SN<br>Infiltration of CD4+Foxp3+ Treg cells in nigrostriatal system | (Sanchez-Guajardo, Annibali, et al. 2013) |
| **AFFITOPE®-KLH peptide (PD1 and PD3)** | Mouse, mThy1.2–a-Syn, Line 61 | Induction of an aSyn selective IgG Ab response (aSyn recognized, β-Synuclein spared)<br>Generated Abs pass the BBB and bind to aSyn deposits<br>Reduction of pathological aSyn (oligomers, aggregates)<br>Reduction of model-specific neuropathological alterations<br>Improvement in motoric function (body suspension test) | (Mandler et al. 2014) |
| **aSyn peptide-TT P30 conjugate** | Mouse,Wt-C57Bl/6 | All 3 conjugates induce human aSyn–specific Abs (aSyn 85-99, aSyn109-126, aSyn126-140)<br>T cell responses to P30, but not to human aSyn (aSyn 109-126-TT P30 conjugate)<br>Generated Abs h aSyn in brain extracts(aSyn 126-140-TT P30 conjugate) | (Ghochikyan et al. 2014) |

Given the already stablished relationship between aSyn and the activation of the IS, several groups have developed immune therapies that primaryly target aSyn species (Schneeberger et al. 2016)(**Table2**). Indeed various studies performed following pioneering work by Masliah et al. (Masliah et al. 2005) have explored different types of approaches for immunization with aSyn in animal models. Overall, these different approaches can be subdivided into three 'schools'. The first one uses the immunization strategy to obtain a potent humoral response against aSyn species, coupled with negligible or even absent cellular response(Masliah et al. 2005; Mandler et al. 2014; Ghochikyan et al. 2014). Although these approximations have shown promising results in mouse models, such as reduction of aSyn aggregates, and improvement in motor function, direct translation to clinics still needs to be assessed due to possible break of self-tolerance (Schneeberger et al. 2016). Importantly, two formulations based on this principle are now under clinical trials, after showing promising results in 3 animal models. AFFITOPE technology uses small peptides that mimic the target epitope as vaccine antigens, avoiding the Tcell respose and the problem of braking self-tolerance but capable of generate a long-lasting, sustained antibody response (Mandler et al. 2014). Currently two AFFITOPE fomulations, PD01 and PD03, are now being tested for their tolerability and safety properties (Identifiers: NCT01885494, NCT02267434). In an opposite direction Gendelman and co-workers have developed different approximations in order to promote a cellular response, in particular an antigen-specific regulatory T cell (Treg) response, to try restore the immune imbalance in PD (Benner et al. 2008; Reynolds, Kadiu, et al. 2008; Olson & Gendelman 2016). Between these two opposite approximations we find the work of Sanchez-Guajardo and co-workers. Actually they found that immunization of AAV rat model with full-length aSyn displayed increased aSyn specific antibodies coupled with an increase in Tregs, and interestingly this

immune response was accompanied by a reduction in aSyn aggregates (Sanchez-Guajardo, Annibali, et al. 2013). In conclusion, despite the promising results showed shown by these different approximations, neither approach on its own has shown to be sufficient to counteract the multiple pathogenic avenues in PD, most notably the uncontrolled neuro-inflammatory state linked to neurodegeneration, and further, their therapeutic efficacy in human subjects still needs to be tested. Moreover, an important limitation when designing immunotherapeutic strategies against PD is the insufficient state of knowledge about the activation and evolution of the immune response linked to PD along the course of disease and which immune profiles are beneficial, and which ones are detrimental, for PD progression.

## 4.2 CHAPEROME IN IMMUNOTHERAPY

The eukaryotic 'chaperome' has been defined as an interconnected network composed of a large number of molecular chaperones and co-chaperones that are essential for protein homeostasis (Brehme *et al.*, 2014). Indeed, the main or best known functions of this kind of proteins is to chaperone nascent polypetides to assist *de novo* protein folding and to prevent protein misfolding and aggregation under heat-shock or several other environmental stresses (Albanèse *et al.*, 2006). Accordingly, the members of the chaperome are the most abundant proteins inside the cell while their presence in the extracellular space is a well-known DAMPs (Miyake and Yamasaki, 2012; Y Tamura *et al.*, 2012; Yasuaki Tamura *et al.*, 2012). In fact, several heat-shock proteins (HSPs) have lately been reported to play diverse roles as modulators of innate and adaptive immunity (Binder, 2014; Tosti *et al.*, 2014; Radons, 2016). Firstly, it was established that different members of the HSP70 and HSP90 families of chaperones, such as hsp70 (HSPA1A), and gp96/grp94 are necessary for the correct processing and presentation of antigens in APCs along with the MHC-I

and MHC-II histocompatibility complexes (Binder, 2014). Lately, different studies have shown that extracellular HSPs can act as danger signals (DAMPs) and actually different APCs express, on their surface, receptors capable of recognizing specific HSPs (Binder, Han and Srivastava, 2000; Basu *et al.*, 2001; Binder, 2014). Since then, several immune-modulating functions have been described for different members of the chaperome, from promoting antigen cross-presentation and maturation of dendritic cells to exerting immunosuppressive signals, or facilitating the activation of lymphocytes and macrophages (Murshid, Gong and Calderwood, 2012; Y Tamura *et al.*, 2012; De Maio and Vazquez, 2013; Muralidharan and Mandrekar, 2013).

As a result of these novel immunomodulatory activities of HSPs, different HSPs have been studied for their treatment feasibility in certain diseases. First of all Srivastava and coworkers demonstrated that immunization in mice with gp96/grp94 (an endoplasmic reticulum-resident chaperone member of the Hsp90 family) isolated from tumours was capable of providing resistance against further challenges with the same tumours (Srivastava, DeLeo and Old, 1986). Surprisingly, the specificity of the elicited immune response was shown to be due to a differential peptide repertoire chaperoned by gp96. After that, a few other HSPs have been tested for their chaperone and anti-tumour capabilities. For instance hsp70, hsp90, hsp110 or grp170 have shown their anti-tumour activity in different animal models (Udono and Srivastava, 1993, 1994; Wang *et al.*, 2001). The use of chaperones in immune-therapeutic approaches has also been explored for treatment of autoimmune diseases, such as Rheumatoid Arthritis (RA), as well as in a variety of infections (Srivastava, 2012; Binder, 2014). Interestingly, in the case of RA, immunotherapeutic use of hsp40, hsp70, hsp90 or BiP, have shown their

capacity to induce antigen-specific T cell suppression activity in different RA murine animal models (Van Herwijnen *et al.*, 2013; Panayi and Corrigall, 2014).

Therefore, HSPs and probably other chaperone proteins possess highly valuable features that could be potentially exploited to neutralize or redirect the abnormal, neurodegeneration-linked immunity elicited by misfolding proteins in misfolding/amyloid disorders, namely, their classical 'chaperone' functions in combination with their proved immunomodulatory activities.

# CHAPTER 1:

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## Preconditioning of Microglia by α-Synuclein Strongly Affects the Response Induced by Toll-like Receptor (TLR) Stimulation

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# OBJECTIVES

Along the past decade the relationship between the accumulation of abnormal α-synuclein species, microglial activation and the pathophysiology of several neurodegenerative diseases, including Parkinson's disease, has been demonstrated. However, the exact mechanisms underlying its pathological actions are poorly understood. On the other hand, the finding through epidemiological studies and animal models, of a link between specific microbial infections and the occurrence of these neurological disorders point to a possible role of the TLR system in the onset and progression of these diseases. In order to further investigate this question we aimed to:

Study the impact of extracellular aSyn priming on the microglial innate immune response following TLR stimulation.

# Preconditioning of Microglia by α-Synuclein Strongly Affects the Response Induced by Toll-like Receptor (TLR) Stimulation

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## 1. Abstract

In recent years, it has become accepted that α-synuclein (aSyn) has a key role in the microglia-mediated neuroinflammation, which accompanies the development of Parkinson's disease and other related disorders, such as Dementia with Lewy Bodies and Alzheimer's disease. Nevertheless, the cellular and molecular mechanisms underlying its pathological actions, especially in the sporadic forms of the diseases, are not completely understood. Intriguingly, several epidemiological and animal model studies have revealed a link between certain microbial infections and the onset or progression of sporadic forms of these neurodegenerative disorders. In this work, we have characterized the effect of toll-like receptor (TLR) stimulation on primary murine microglial cultures and analysed the impact of priming cells with extracellular wild-type (Wt) aSyn on the subsequent TLR stimulation of cells with a set of TLR ligands.

By assaying key interleukins and chemokines we report that specific stimuli, in particular Pam3Csk4 (Pam3) and single-stranded RNA40 (ssRNA), can differentially affect the TLR2/1- and TLR7-mediated responses of microglia when pre-conditioned with aSyn by augmenting IL-6, MCP-1/CCL2 or IP-10/CXCL10 secretion levels. Furthermore, we report a skewing of aSyn-primed microglia stimulated with ssRNA (TLR7) or Pam3 (TLR2/1) towards intermediate but at the same time differential, M1/M2 phenotypes. Finally, we show that the levels and intracellular location of activated caspase-3 protein change significantly in aSyn-primed microglia after stimulation with these particular TLR agonists. Overall, we report a remarkable impact of non-aggregated aSyn pre-sensitization of microglia on TLR-mediated immunity, a phenomenon that could contribute to triggering the onset of sporadic α-synuclein-related neuropathologies.

## 2. Introduction

The synucleinopathies are a group of pathologies increasingly affecting the population over 65 years old, comprising various progressive, neurodegenerative disorders including Parkinson's disease (PD), dementia with Lewy bodies (DLB) and multiple system atrophy (MSA) (Fellner & Stefanova 2012). Despite the particular characteristics regarding the type of cells and brain areas affected, these disorders have in common the accumulation of α-synuclein (aSyn) insoluble aggregates as the main pathological feature (Marques & Outeiro 2012). Moreover, aSyn has also been identified as a component of amyloid brain tissues in AD patients (Hamilton 2000). PD, the most prevalent of these pathologies, is characterized pathologically by the presence of intraneuronal inclusions highly enriched in aSyn (known as Lewy bodies) in the substantia nigra (SN) of the brain (Spillantini et al. 1998; Croisier et al. 2005; Chiti & Dobson 2006), and the loss of dopaminergic neurons

(Moore et al. 2005). Three missense mutations of aSyn, A30P, E46K, and A53T, as well as multiple copies of the wildtype (Wt) gene, are linked to rare, early onset PD cases (Moore et al. 2005). Even though aSyn is now recognized as a key player in the pathogenesis of PD and other aSyn-related disorders, the cellular and molecular events underlying its pathological actions are not understood in detail. Moreover, the underlying mechanisms driving the development of sporadic PD and other synucleinopathies and which constitute the vast majority clinical cases, remain largely unknown (Marques & Outeiro 2012; Cookson 2009).

Accumulated evidence shows that inflammation is involved in the pathogenesis of a number of neurodegenerative diseases including PD, AD, and multiple sclerosis (MS), among others (Kim & Joh 2006). Indeed, it is now well established that the onset and progression of PD is accompanied by a robust inflammatory response essentially mediated by activated microglia in affected areas of the brain, which is thought to precede neuronal degeneration (Gao et al. 2008; Long-Smith et al. 2009; Marinova-Mutafchieva et al. 2009). Moreover, several in vitro studies have revealed the ability of exogenous aSyn, especially the PD-linked mutational variants and the oligomeric forms of aSyn, to induce such a response by stimulated microglia (Fellner et al. 2011; Roodveldt et al. 2010; Roodveldt et al. 2011). This finding is particularly important as the presence of aSyn has been detected in extracellular biological fluids including human cerebrospinal fluid (CSF) (Borghi et al. 2000; El-Agnaf et al. 2003; El-Agnaf et al. 2006; Emmanouilidou et al. 2011) and also shown to be secreted from neuronal cells (Emmanouilidou et al. 2011; Lee et al. 2005; Lee 2008). Importantly, imbalances in Wt extracellular aSyn levels –both aggregated and monomeric- have been detected in CSF from patients with PD, AD, DLB and certain forms of prion diseases (Mollenhauer et al. 2008; Mollenhauer et al.

2011; Mollenhauer et al. 2013; Tokuda et al. 2006; Tokuda et al. 2010). Even though it is unclear to what extent such variations in CSF mirrors the concentrations within key brain regions and whether they arise at the very initial stages of disease, they indicate that non-aggregated extracellular aSyn within the local environment could be highly relevant for pathogenesis.

Intriguingly, several epidemiological and animal studies have revealed a link between certain bacterial, viral and parasitic infections and the development or progression of sporadic PD (Takahashi & Yamada 1999; Charlett et al. 1999; Tsui et al. 1999; Weller et al. 2005; Jang et al. 2009; Dobbs et al. 2010; Miman et al. 2010) and AD (Krstic et al. 2012; Hammond et al. 2010; Balin et al. 2008). Nevertheless, the mechanism by which infectious agents or inflammatory stimuli could exacerbate or modulate these microglial phenotypes is not well understood.

Activation of microglia and inflammation in the context of PD, amyotrophic lateral sclerosis (ALS), and AD is currently thought to involve the toll-like receptors (TLRs) (Lehnardt 2010; Watson et al. 2012; Béraud & Maguire-Zeiss 2012), a group of transmembrane proteins known to detect invading pathogens (Kumar et al. 2011). TLRs have been reported to be up-regulated in α-synucleinopathy-derived brain tissue from mouse and human subjects (Stefanova et al. 2007; Letiembre et al. 2009), and have been proposed to mediate different pathways leading to either neuroprotective or neurotoxic phenotypes (Letiembre et al. 2009; Block et al. 2007; McKimmie et al. 2006; Carty & Bowie 2011). Although it has recently been shown that treatment of cells with aggregated aSyn is able to alter the TLRs gene expression in microglial cells (Beraud et al. 2011), there are as yet no reported studies on the cellular mechanisms modelling a pre-oligomeric stage of the disease that could recapitulate the setting of neuroinflammation and neurodegeneration. This fact

highlights the importance of studying the impact of extracellular aSyn on the innate immune response following TLR stimulation. In this work, by characterizing the effect of a set of TLR agonists on primary microglia cultures, we report a remarkable impact of Wt aSyn priming of cells on TLR mediated immunity that might reflect a causal link between certain infections and the initiation of sporadic neurodegenerative disease.

## 3. Material and Methods

### TLR ligands

TLR ligands were purchased from InvivoGen (San Diego, USA) and prepared according to the manufacturer's recommendations. The TLR ligands were: bacterial lipopolysacharyde (LPS), type B CpG oligonucleotide ODN 1688 (CpG), low molecular weight polyinosine-polycytidylic acid (poly(I:C-LMW)) (PolyI:C), lipoteichoic acid from B. subtilis (LTA), synthetic bacterial lipoprotein Pam3CSK4 (Pam3), peptidoglycan from B. subtilis (PGN), imiquimod (Imiq), and ssRNA40/LyoVec (ssRNA).

### α-synuclein protein overexpression, purification and characterization

Human Wt aSyn was overexpressed in E. coli BL21(DE3) cells using plasmid pT7-7 and purified as described previously (Roodveldt et al. 2010). The purity and monomeric state of the aSyn preparation (>95%) was assessed by 15% SDS-PAGE, mass spectrometry analysis, and 4-10% native PAGE (Lonza, Basel, Switzerland), as previously described (Roodveldt et al. 2010). The preparation and characterization of soluble aSyn oligomers was carried as reported previously (Roodveldt et al. 2012) and purified oligomeric fractions were stored at 4 °C for up to 24 hrs. Endotoxin levels in the protein preparations were

measured by the ToxiSensor Chromogenic LAL Assay Kit (GenScript, Piscataway, USA), and values obtained were <1 EU/mg protein in all cases. The protein concentration of non-aggregated and oligomeric aSyn was determined by means of Micro BCA Reagent Kit (Pierce, Rockford, IL, USA).

**Preparation and characterization of primary microglial cell cultures**

Mixed glial cultures were prepared from cerebral cortices of 1-3 day-old C57BL/6 male mice (University of Seville Animal Core Facility, Seville, Spain), and the microglial fraction was isolated, according to previously described methods (Roodveldt et al. 2010). Purified microglial cells were characterized by immunocytochemistry as cells of the haematopoietic lineage on the basis of their expression of the pan haematopoietic marker CD45 and of the monocyte/macrophage markers CD11b, F4/80 and CD68, as described elsewhere (Roodveldt et al. 2010). Additionally, the absence of glial fibrillary acidic protein (GFAP)-positive astrocytes in purified microglial cell cultures was also assessed according to a previously reported method (Roodveldt et al. 2010).

**Treatment of microglial cell cultures**

Stimulation of microglial cultures in 12-well plates was performed by replacing the medium by adding 1 mL Wt aSyn (or medium alone in the case of the following controls: 'untreated microglia' and 'TLR ligand alone' controls), at a final concentration of 1 μg/mL (equivalent to 70 nM) diluted in complete DMEM-F12 medium. After a 6-hour incubation at 37°C, 110 μL aliquot of TLR ligand solutions (at 10x higher concentration) in complete DMEM-F12 medium was added to each well containing either aSyn-primed, or non-primed cells. In the case of some controls, 110 μL of medium alone was instead added to the

wells. The final concentrations of the ligands being tested correspond to conditions reported previously (Castillo et al. 2008), and were: LPS, 1 µg/mL; CpG, 1 µg/mL; Poly(I:C), 50 µg/mL; LTA, 10 µg/mLl; Pam3, 1 µg/mL; PGN, 10 µg/mL; Imiq; 1 µg/mL; and ssRNA, 0.25 µg/mL. Cell culture samples with aSyn-primed cells but with no subsequent TLR ligand stimulation, as well as cells with no addition of aSyn or TLR ligands, were used as controls. Cells were then incubated for a further period of 18 hrs. In addition, some controls were prepared in parallel by incubating cells for 24 hrs with 1 µg/mL A30P aSyn or oligomeric Wt aSyn. In all cases, after incubation for a total of 24 hrs, the supernatants were harvested and the cells were frozen and stored at -80 °C.

**Cytokine release measurements**

After treatment and incubation of cells for a total of 24 hrs as explained in the previous subsection, culture supernatants were harvested and centrifuged at 700 g for 5 min. The supernatants from treated cultures were recovered and stored at -80 °C before measurement of cytokine levels. IL-6, TNFα, IL-1ß, IL-10, IL-13 and IL17 levels were assayed using the mouse IL-6/IL-10 BD OptEIA ELISA set (BD Biosciences, Franklin Lakes, NJ, USA), the murine IL-13 ELISA Development Kit (Peprotech, London, UK), and the mouse IL-17 DuoSet® ELISA Development System (R&D Systems, Minneapolis, USA), according to the manufacturer's protocols. Chemokine levels in the culture supernatants were determined by a specific sandwich ELISA by using capture/biotinylated detection antibodies obtained from Peprotech (London, UK) according to the manufacturer's recommendations.

**Determination of TLR gene expression**

Expression levels of the genes for TLRs 1, 2, 3, 4 and 7, and for hypoxanthine-phophoribosyltransferase (HPRT), were determined by using a two-step quantitative real-time PCR (qRT-PCR) method. Total RNA from treated microglial cells was extracted using the Tripure Isolation Reagent (Roche, Basel, Switzerland) according to the manufacturer's protocol. RNA (1 µg) was reverse-transcribed by using the Quantitect Reverse Transcription kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. qRT-PCR was performed with SensiFAST™ SYBR Lo-ROX Kit (Bioline, London, UK) on an ABI Prism 7500 Real Time PCR System. Primer pairs were designed to anneal in different exons, and were: HPRT_For: 5'-GTAATGATCAGTCAACGGGGGAC-3', HPRT_Rev: 5'-CCAGCAAGCTTGCAACCTTAACCA-3'; primers for TLR genes were purchased from Sigma (Sigma- Aldrich, St. Louis, USA). TLR4_For: 5'-ACCAGGAAGCTTGAATCCCT-3'; TLR4_Rev: 5'-TCCAGCCACTGAAGTTCTGA-3'; TLR7_For: 5'-TCAAAGGCTCTGCGAGT-3'; TLR7_Rev: 5'-AGTCAGAGATAGGCCAGGA-3'. TLR1, TLR2 and TLR3 primers were purchased from Qiagen (Hilden, Germany). Multiple transcripts were analysed simultaneously for 40 cycles using an optimized qRT-PCR thermal profile. Changes in gene expression were determined using the ΔCt value taking hprt as endogenous control. The ΔΔCt values were calculated by subtracting ΔCt values of non-primed samples followed by TLR stimulation ('TLR ligand') from samples treated with aSyn priming followed by TLR stimulation ('Wt+TLR ligand').

**Phagocytosis assays**

FluoresbriteTM carboxylate microspheres of 0.75 µm diameter (2.64 % Solid-Latex; Polysciences Inc, Warrington, USA) were used as fluorescein-conjugated tracker microparticles for measuring the phagocytosis capacity of differentially

activated microglial cells. 1 hr before starting the phagocytosis assay, FITC-labelled microspheres (1.08 x 1011 particles/mL) were mixed at a ratio of 1 µL microspheres: 20 µL FBS for 1 hr at 37°C into inactivated FBS (BioWhittaker, Verviers, Belgium) and incubated for a further 1 hr at 37 °C in order to opsonise fully the carboxylate groups. The mixtures of microspheres and FBS were then resuspended in fresh DMEM-F12 medium (BioWhittaker, Verviers, Belgium), with L-glutamine and P/S antibiotics supplements to obtain normal 10% FBS supplemented media containing 5.4 x 108 microspheres/mL. After removal of 500 µL of supernantant from the aSyn-stimulated microglial cell cultures for cytokine release analyses, a volume of 150 µL of resuspended microspheres was added to the remaining 600 µL in each well to obtain a final concentration of $1x10^8$ particles/mL. Particles were then homogenously distributed throughout the well by gentle movement of the plate and incubated for 1 hr at 37 °C. Medium containing non-phagocytosed microspheres was then removed and the cells were washed with PBS prior to fixation with 4% p-formaldehyde in PBS for 30 min at 4 °C. A volume of 1 mL of PBS containing the nuclear fluorescent dye bisBenzimide H33342 tri-hydrochloride (Hoechst 33342; 1 µg/mL) was then added to the cells, and the plates were stored at 4 °C for a minimum of 24 hrs until being analysed. For this purpose, an Olympus IX71 fluorescence microscope equipped with the digital image processing software DPController and DPManager (Olympus Europa, Hamburg, Germany), was used. For each sample, the phagocytic capacity of microglial cells was determined by analysing fluorescent images of phagocytosed FITC-labelled microspheres and Hoechst stained nuclei from four randomly chosen fields (each containing ~85 cells). For each random field, the total numbers of spheres and nuclei were determined using the Granularity application of the digital imaging analysis software Metamorph (MDS Analytical Technologies, Toronto, Canada), and the number of spheres per nucleus, as an indicator of the

phagocytic capacity, was calculated for every field analysed. The values shown correspond to the mean from two or three independent experiments (N=2 or 3), each one containing duplicate samples.

**Determination of Arg1/iNOS gene expression**

Total RNA from treated microglial cells was extracted and reverse-transcribed as described before in this section. The primers used were as follows: for mouse arginase-1/Arg1 (band size: 264 bp): Forward: 5´-CAGAAGAATGGAAGAGTCAG-3´; Reverse: 5´-CAGATATGCAGGGAGTCACC-3´, for mouse iNOS (band size: 373 bp): Forward: 5´-GCCTCATGCCATTGAGTTCATCAACC-3´; Reverse: 5´-GAGCTGTGAATTCCAGAGCCTGAAG-3´, and for mouse actin (band size: 165 bp): Forward: 5´- TGTTACCAACTGGGACGACA-3´; Reverse: 5´-GGGGTGTTGAAGGTCTCAAA-3´. DNA Marker: Fermentas*phiX174 DNA/BsuR I (Hae III) Marker, 9.

The positive controls for the Arg and iNOS PCR assays (PCR+) were bone marrow derived macrophages either non stimulated or stimulated 24 hrs with IL-4 (10 ng/mL), respectively. Macrophages were isolated from bone marrow from Balb/c mice and cultured as follows: bone marrow cells (0.4 x 106/mL) were cultured in DMEM (2 mM L-glutamine, 100 units/mL penicillin/streptomycin and 20% heat-inactivated FCS, all from Gibco/Invitrogen) containing 20 ng/mL M-CSF (Peprotech) for 7-8 days. Differentiated macrophages were detached by incubating the plates with 2 mM EDTA/PBS at 37 °C for 10 min. Cell preparations typically consisted of >95% CD11b+CD11c- macrophages. Bone marrow derived macrophages were plated at 8 x 105 cells/well in 6-well plates. After 4 hrs of adherence, macrophages

were washed with PBS and stimulated with IL-4 (BD Bioscience; 10 ng/mL) for 24 hrs.

Final concentrations in the PCR reaction mixture were: cDNA template: 60 ng; dNTPs: 0.2 mM; primers: 0.4 µM; MgCl2: 2mM; Taq polimerase (Biotools): 0.625 units/reaction, in a total volume of 25 µL. PCR conditions were as follows: for Arg1: 94°C-5 min; 94°C-30 sec, 56°C-30 sec, 72°C-30 sec (30 cycles); 72°C-7 min; 4°C-o/n. For iNOS: 94°C- 35 sec; 62°C-2min; 72°C-2 min (35 cycles); 72°C-7 min; 4°C-o/n. For actin: 94°C-5 min; 94°C-30 sec, 60°C-30 sec, 72°C-30 sec (30 cycles); 72°C-7 min; 4°C-o/n.

**Determination of cleaved caspase-3 levels by ELISA**

For detecting activated caspase-3 protein levels by ELISA, the Human/Mouse Cleaved Caspase-3 (Asp175)-DuoSet ELISA kit (R&D Systems, Abingdom, UK) was used. For this, total protein was extracted from treated microglial cells in culture with 'lysis buffer' according to the manufacturer's instructions, and quantified with the BCA Protein Assay Reagent Kit (Thermo Fisher Scientific Inc., Rockford, USA).

**Determination of cleaved caspase-3 levels by immunofluorescence**

For immunofluorescence (IF) analysis, highly pure microglial cell cultures were obtained as described above (in the 'Preparation and characterization of primary microglial cell cultures' section), but in this case cultures were prepared on a hydrophilic µ-Dish (Ibidi GmbH, Germany). 3-4 days after isolation, microglial cells were treated as described before (in the 'Preconditioning of microglial cell cultures with aSyn and stimulation with TLR ligands' section). For the immunolabelling step, treated microglial cultures were

thereafter fixed in cold PBS containing 4% p-formaldehyde for 15 min at 4 °C and then washed in PBS prior to being permeabilized and blocked in PBS containing 3% BSA (Sigma-Aldrich, St. Louis, USA) and 0.5 % Triton X-100 (Sigma-Aldrich, St. Louis, USA) for 1 hr at 4°C. Cells were incubated o/n at 4 °C with cleaved caspase-3 (Asp175) antibody (Cell Signalling Technology Inc., Danvers, USA) at a 1/200 dilution in PBS containing 3% BSA and 0.5% Triton X-100. After 3 washes with PBS, cells were incubated for 1 hr at room temperature in the dark with a donkey anti-rabbit IgG-AlexaFluor 594 secondary antibody (Invitrogen, Paisley, UK) at a 1/800 dilution in the same buffer. After three washes with PBS, the nuclei were counterstained by incubating cells with PBS containing 1 µg/mL Hoechst 33342 (Sigma-Aldrich, St. Louis, USA) for 1 hour at 4 °C and examined under the fluorescence microscope. Fluorescence images were captured with an Olympus IX71inverted fluorescence microscope equipped with digital image processing softwares DPController and DPManager (Olympus. www.olympus.co.uk). Fluorescence images were taken at x20 magnification from randomly chosen fields. A rigorous comparative evaluation of cleaved caspase-3 immunoexpression was achieved by taking fluorescence images with the same exposure time. Fluorescence images (cleaved caspase-3 marker and nuclear Hoechst 33342 stainings) taken for different microglial cell cultures were finally merged at the same ratio with the use of DPManager software. Phase-contrast images were also obtained from the same cultures, at x10 magnification. For quantitative analysis of cleaved caspase-3 levels by IF, the imaging software MetaMorph Offline (version 7.5.1.0, MDS Analytical technologies, USA), was used. Analysis was performed by using the ratio of the intensity values of Alexa 594 nm (RF) and Hoechst 33342 (BF) above background (areas lacking cells), and the data were expressed as arbitrary units (AU) and exported automatically from to Microsoft Excel program through a summary log. For measuring the relative

cleaved caspase-3 levels per cell, the total specific RF/BF (red fluorescence/ blue fluorescence) ratio was calculated.

**Data analysis**

All values are expressed as the mean ± S.E.M. Statistical significance was evaluated by the Student's t-test using SPSS Statistics 19.0 (IBM Company, Chicago, USA). Statistically significant differences in relative cytokine release levels, phagocytosis and TLR expression [(Wt+TLR ligand): (TLR ligand) fold-changes] were calculated with the t-Student test between two sets of results by comparing the values under conditions of aSyn-preconditioning followed by TLR stimulation (Wt+TLR ligand) relative to conditions in the absence of aSyn preconditioning and TLR stimulation (TLR ligand). Each set of results originated from several independent experiments (N=3-7). In addition, in the cases of statistically significant changes, the absolute values were also compared by applying a two-way Student's t-test for each (Wt+TLR ligand) sample condition to those resulting from stimulation of cells with aSyn alone ('Wt aSyn') or untreated cells ('Control'). For quantitative analysis of cleaved caspase-3 levels by IF, three images from random fields (N=3), each containing 80-90 cells, were used to calculate the mean RF/BF value and SEM for each sample condition. For quantitative ELISA assays, results originated from three independent experiments (N=3). Statistically significant differences of results from IF and ELISA assays were calculated by applying the Student's t test in relation to the values obtained with the corresponding TLR ligand in the absence of aSyn-preconditioning and with Wt aSyn alone. In all cases, statistically significant differences between the two sets of results were those with $p<0.05$.

**Ethics Statement**

All animals were handled in strict accordance with good animal practice as defined by the relevant national/EU guidelines and The CEA-CABIMER Experimental Animal Committee, and all animal work was approved by the appropriate committee (file CEA-2010-14).

## 4. Results

**Priming of microglia with Wt aSyn primarily affects the TLR2 and TLR7-mediated immune response**

Given that the underlying mechanisms initiating and accompanying the development of sporadic synucleinopathies remain essentially unknown, we decided to carry out our studies with Wt aSyn protein. Although various types of cells have been identified as a source of cytokines in the central nervous system (CNS), microglia appear to be a principal source of pro-inflammatory and immune regulatory cytokines (Kim & Joh 2006). In order to explore the possible impact of aSyn as a priming factor in the microglial immune response following pathogen invasion, we challenged Wt aSyn-primed microglial cultures with a set of TLR agonists, namely Pam3Csk4 lipopeptide (Pam3; TLR2/1), peptidoglycan from B. subtilis (PGN; TLR2), lipoteichoic acid from B. subtilis (LTA; TLR2), Poly(I:C)-LMW—a synthetic analog of dsRNA- (PolyI:C; TLR3), bacterial lipopolysaccharide (LPS; TLR4), imiquimod —a small synthetic antiviral molecule- (Imiq; TLR7), ssRNA40 oligonucleotide complexed with LyoVec (ssRNA; TLR7), and type B CpG oligonucleotide (CpG; TLR9). We omitted the study of TLR5 stimulation based on previous reports of its absence in mouse microglia (McKimmie et al. 2006; Applequist et al. 2002), and on our own

observations of a lack of effect with the TLR5 ligand flagellin as measured by secreted levels of proinflammatory cytokines (data not shown).

After incubation with 1 μg/mL (equivalent to ca. 70 nM) of non-aggregated Wt aSyn for 6 hrs (or mock solution in the case of non-preconditioned samples and untreated controls), the cell cultures were incubated for a further 18 hrs with standard concentrations of different TLR agonists, or otherwise treated with medium alone to serve as controls. The aSyn working concentration was chosen considering the typical range from previous reports and especially based on our earlier work in which a similar experimental setup has been used (Roodveldt et al. 2010). After incubation of cells for a total of 24 hrs, the supernatants were recovered for later analysis of their interleukin/chemokine contents. Given that the aggregated aSyn as well as the A30P aSyn variant have been previously shown to exert a stronger pro-inflammatory effect on microglia (Roodveldt et al. 2010; Zhang et al. 2005), control samples were prepared by incubating cells for 24 hrs with 1 μg/mL A30P aSyn variant or 1 μg/mL oligomeric aSyn species.

A set of key interleukins, namely pro-inflammatory IL-6, immunoregulatory IL-10, anti-inflammatory IL-13, and autoimmunity-related IL-17, were assayed by ELISA (**Table 1**). Our results show that, at the concentrations used, all eight TLR ligands, as well as A30P and oligomeric aSyn, produced higher IL-6 secretion when added alone to microglial cells, relative to untreated controls (**Table 1**). In addition, stimulation with LPS and Poly I:C caused an increase in IL-10 and IL-13 levels, respectively, while oligomeric aSyn appeared to cause a reduction in IL-13 secreted levels (**Table 1**).

| | IL-6 (pg/mL) | IL-10 (pg/mL) | IL-13 (pg/mL) | IL-17 (pg/mL) |
|---|---|---|---|---|
| **Control** | 64.4 ± 10.3 | 120 ± 34,4 | 44.1 ± | 8.6 ± 1.1 |
| **Wt aSyn** | 198 ± 89.8 | 20.8 ± 12.9 | 72.3 ± | 9.2 ± 0.8 |
| **A30P aSyn** | 1880 ± 604 | 150.2 ± | 59.0 ± | 11.6 ± 4.4 |
| **Olig. Wt aSyn[a]** | 481 ± 130 | N/A | 33.1 ± | N/A |
| **LPS** | 7719 ± | 253 ± 37.5 | 61.6 ± 8.1 | 9.8 ± 1.4 |
| **CpG** | 1542 ± 296 | 182 ± 45.4 | 65.6 ± | 9.0 ± 1.2 |
| **Pam3** | 1604 ± 283 | 111.4 ± | 47.8 ± 7.4 | 11.0 ± 2.0 |
| **Imiq** | 1041 ± 235 | 66.5 ± 18.6 | 48.1 ± | 8.9 ± 1.2 |
| **ssRNA** | 256 ± 40 | 68.5 ± 26.1 | 66.8 ± | 9.9 ± 1.4 |
| **PGN** | 1037 ± 379 | 63 ± 25.6 | 61.0 ± | 7.9 ± 0.9 |
| **LTA** | 1731± 297 | 118.5 ± | 66.7 ± | 7.9 ± 1.2 |
| **Poly I:C** | 1641 ± 541 | 64 ± 23.9 | 178.0 ± | 7.9 ± 1.0 |

**Table 1**. **Values of secreted interleukins in primary microglia 24 hrs after treatment.** Following treatment of cells with aSyn or with TLR agonists at the concentrations described in the Materials and Methods section, cell culture supernatants were harvested after incubation for 24 hrs, and cytokine levels were assayed by ELISA. Values correspond to the mean of six independent experiments (N=6) each containing duplicate samples and error corresponds to SEM, except for (a , in which the values shown are the mean of two independent experiments (N=2) each containing four replicas and the error corresponds to SD. doi: 10.1371/journal.pone.0079160.t001

We then sought to assess the impact of aSyn-preconditioning of microglia on the interleukin secretion profile (**Figure 1A**). A general increase trend in TNFα secreted levels could be observed for the primed cells after TLR stimulation, in particular with (TLR7) ssRNA, (TLR2/1 Pam3) and (TLR2) PGN (**Figure S1**). This effect was not seen for IL-1ß, as only (TLR3) Poly I:C produced an increase trend in its secretion levels under aSyn priming conditions (**Figure S1**). On the other hand, a significant 4-fold increase in IL-6 release levels was observed for the Wt

aSyn-primed cells after stimulation with Pam3 (TLR2/1) (p=0.024), relative to the corresponding controls with the TLR agonists in the absence of priming with αSyn. Of particular note is that neither IL-10 nor IL-17 levels displayed changes in Wt aSyn-primed cells for any of the tested TLR ligands. Interestingly, even though they did not reach statistical significance, moderate fold-change reductions of IL-13 anti-inflammatory cytokine levels were observed in the cases of stimulation of primed microglia with the TLR7 agonists Imiq and ssRNA40 (**Figure 1A**).

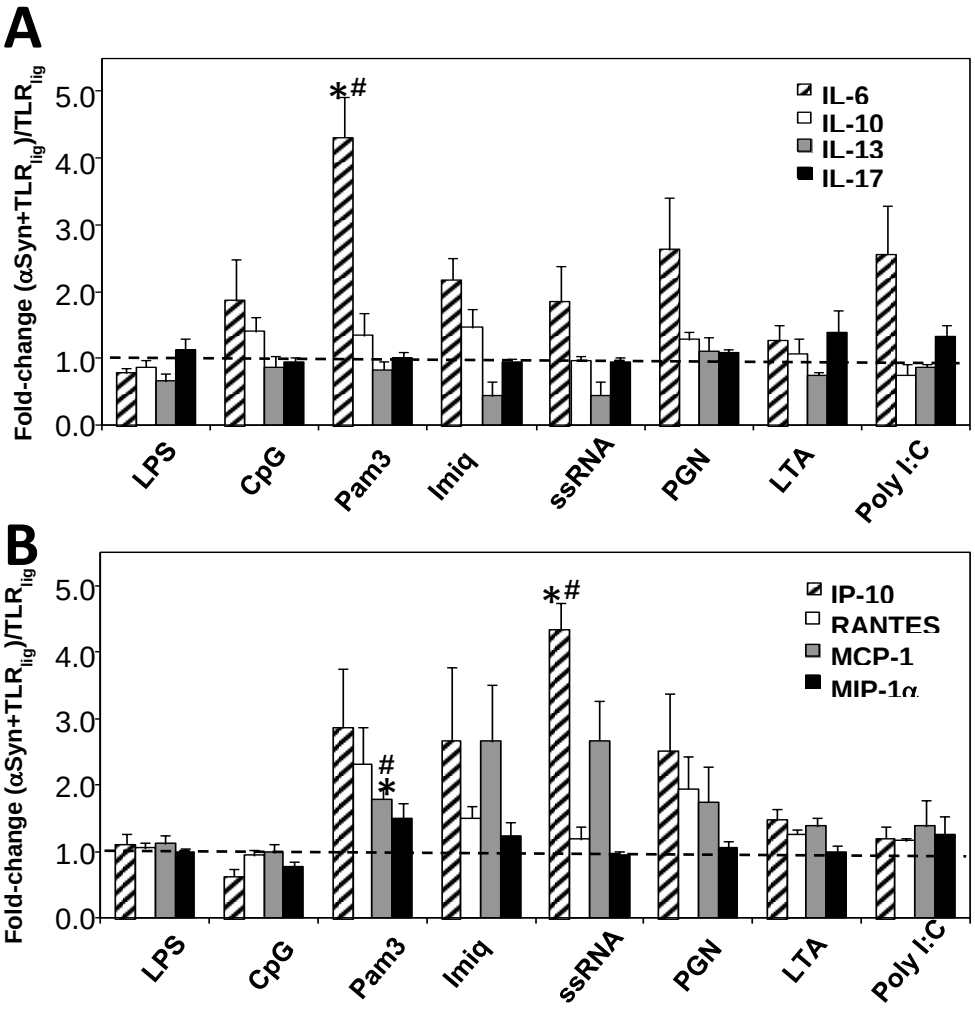


**Figure 1. Impact of Wt aSyn-priming on microglial cytokine release after TLR stimulation**. After treating the microglial cells either with Wt aSyn at 1 µg/mL ('priming' or pre-conditioning) or with 'mock' solution (no pre-conditioning) for 6 hrs, the TLR agonists were added to their specified final concentrations (see Materials and Methods), and incubated for further 18 hrs at 37 °C. The culture supernatants were harvested and used to measure (A) the levels of the interleukins IL-6, IL-10, IL-13, and IL-17, or (B) of the chemokines IP-10/CXCL10, RANTES/CCL5, MCP-1/CCL2, by ELISA. Values are the fold-change calculated as the signal ratio of aSyn-primed, TLR-stimulated cells ('aSyn+TLR ligand') relative to non-primed, TLR-stimulated cells ('TLR ligand'). The results shown (mean ± SEM) are the average of several independent experiments (IL-6: N=5-7; IL-10: N=4-6; IL-13: N=3; IL-17: N=2-3; IP-10, RANTES, MCP-1, and MIP-1α: N=3-5), each containing duplicate samples. Statistically significant differences (* $p<0.05$) were calculated by applying the Student t test between the two sets of results, for all the TLR ligands tested. (#) denotes a result that is significantly different from that obtained after treatment of cells with Wt aSyn alone ($p<0.05$).

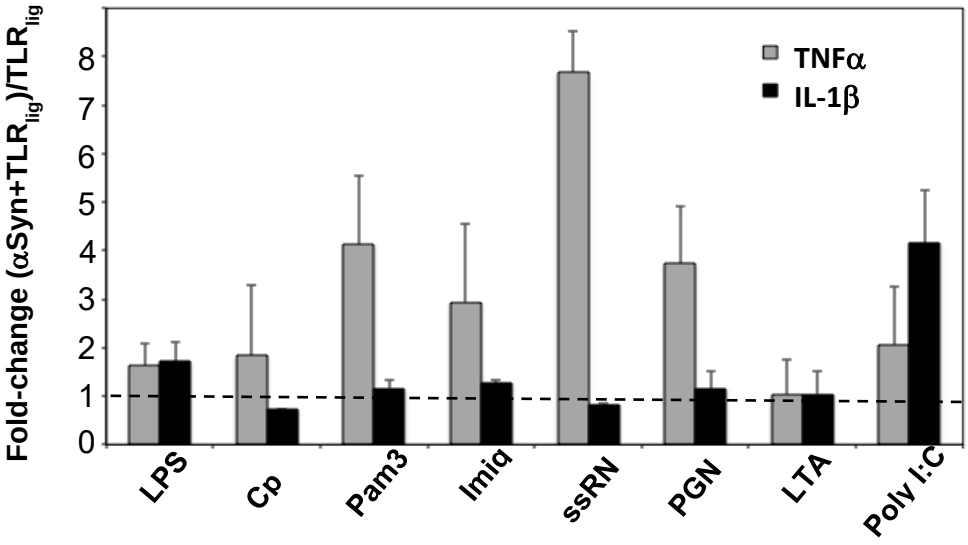



**Figure S1. Impact of Wt aSyn-priming on microglial TNFα and IL-1ß release after TLR stimulation.** After treating the microglial cells either with Wt aSyn at 1 µg/mL ('priming' or preconditioning) or with 'mock' solution (no pre-conditioning) for 6 hrs, the TLR agonists were added to their specified final concentrations (see Materials and

Methods), and incubated for further 18 hrs at 37 °C. The culture supernatants were harvested and used to measure the levels of TNFα and IL-1ß cytokines by ELISA. Values are the fold-change calculated as the signal ratio of aSyn-primed, TLR-stimulated cells ('aSyn +TLR ligand') relative to non-primed, TLR-stimulated cells ('TLR ligand'). The results shown (mean ± SD) are the average from duplicate samples and is representative of three independent experiments for each cytokine measurement. Untreated cells and treatment of cells with Wt aSyn alone were used as controls in both cases. (TIF)

| | IP-10 (pg/mL) | RANTES (pg/mL) | MCP-1 (ng/mL) | MIP-1α (pg/mL) |
|---|---|---|---|---|
| **Control** | 16.9 ± 12.9 | 315 ± 88 | 1.8 0.4 | 11.8 ± 7.2 |
| **Wt aSyn** | 47.2 ± 27.5 | 505 ± 255 | 3.8 ± 1.1 | 4.9 ± 3.3 |
| **A30P aSyn** | 2290 ± 656 | 1887 ± 395 | 11.8 ± 5.9 | 696 ± 276 |
| **Olig. Wt aSyn[a]** | 870 ± 341 | N/A | 6.8 ± 1.7 | N/A |
| **LPS** | 3693 ± 303 | 3967 ± 767 | 27.0 ± 4.6 | 1832 ± 289 |
| **CpG** | 1503 ± 557 | 2183 ± 323 | 14.8 ± 2.8 | 1276 ± 285 |
| **Pam3** | 610 ± 273 | 1289 ± 225 | 15.5 ± 3.9 | 973 ± 75 |
| **Imiq** | 436 ± 192 | 1891± 224 | 18.0 ± 5.0 | 1277 ± 171 |
| **ssRNA** | 209 ± 114 | 609 ± 147 | 4.5 ± 1.1 | 176 ± 105 |
| **PGN** | 549 ± 229 | 722 ± 275 | 9.9 ± 4.8 | 291 ± 92 |
| **LTA** | 2454 ± 490 | 2970 ± 620 | 18.4 ± 3.3 | 1479 ± 287 |
| **Poly I:C** | 3116 ± 460 | 3679 ± 725 | 20.8 ± 2.4 | 943 ± 178 |

**Table 2. Values of secreted chemokines in primary microglia 24 hrs after treatment**. Following treatment of cells with aSyn or with TLR agonists at the concentrations described in the Materials and Methods section, cell culture supernatants were harvested after incubation for 24 hrs, and chemokine levels were assayed by ELISA. Values correspond to the mean of six independent experiments (N=6) each containing duplicate samples and error corresponds to SEM, except for (a, in which the values shown are the mean of two independent experiments (N=2) each containing four replicas and the error corresponds to SD. doi: 10.1371/journal.pone.0079160.t002

We then analysed the secretion profile of treated cells for a set of key chemokines, namely IP-10/CXCL10, RANTES/CCL5, MCP-1/CCL2, and MIP-1α/CCL3 (**Table 2**). Virtually all eight TLR ligands tested, as well as A30P and aggregated aSyn, produced an increase in chemokine secretion for all four chemokines tested when added to cells, relative to untreated controls (**Table 2**). Remarkably, preconditioning of cells with aSyn resulted in a 4.5-fold increase in IP-10 levels upon stimulation with ssRNA (TLR7) ($p=0.015$) (**Figure 1B**). Furthermore, a 2-fold increase in the MCP-1 level was measured for the case of stimulation with Pam3 (TLR2/1) ($p=0.024$), and a similar, although not statistically significant, trend was seen with Imiq and ssRNA (TLR7) (**Figure 1B**).

On the other hand, small and non-significant increases were observed in relative levels of RANTES with LTA (TLR2) or MIP-1α with Pam3, relative levels. Importantly, all of the increases described above that were statistically significant proved to be independent of the sole addition of aSyn (in all cases p values were $<0.05$), implying that these alterations arise from a complex combination of aSyn-priming and subsequent TLR-stimulation effects on microglia.

**aSyn-preconditioning of microglia alters TLR expression when stimulated with Imiq, PGN and Poly I:C**

Given the substantial changes observed in the cytokine secretion profiles as a consequence of the preconditioning process, we sought to investigate whether or not such alterations could result from changes in TLR expression levels. Therefore, we measured the mRNA levels of TLRs 2, 3, 4 and 7 in the samples after treatment as described above, and compared the change in TLR expression in aSyn-preconditioned vs. non-preconditioned, cells. Because stimulation with CpG (TLR9) ligand showed essentially no change trends in

either cytokine release levels as a consequence of priming with aSyn, we omitted further studies with this ligand and continued our characterization with the seven remaining TLR agonists tested (**Figure 2**). On the one hand, significant suppression of TLR7 and TLR3 expression levels were detected in aSyn-preconditioned cells upon stimulation with Imiq (p=0.002) and Poly I:C (p=0.030) ligands, respectively. On the other hand, a modest increase in TLR2 expression was measured aSyn-preconditioned cells stimulated with PGN (p=0.031) (**Figure 2**). However, even though moderate increases in TLR expression were observed for samples stimulated with Pam3 (TLR 2/1), and LTA (TLR2), only a 50% increment in the case of PGN (TLR2) reached statistical significance (p=0.031), while no changes were measured for the cases of LPS and ssRNA (**Figure 2**). These results suggest that targeting receptor-ligand interactions rather, than TLR expression could provide a better rationale and strategy for the management of aSyn-related pathologies.

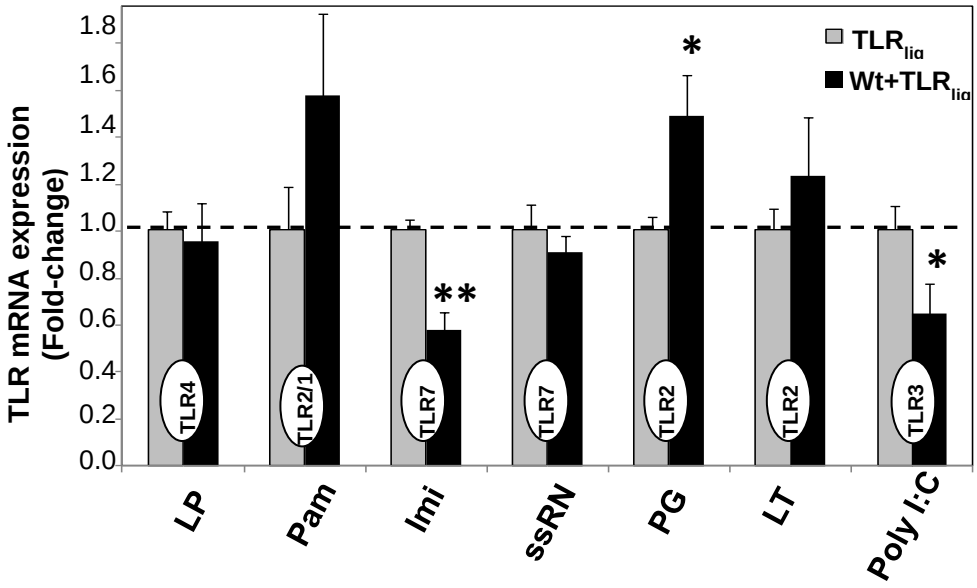



**Figure 2. Comparison of TLR gene expression levels of Wt aSyn-primed vs. non-primed microgia, after TLR stimulation**. After treating cells as before (see legend to Figure 1), cells were lysed and the total RNA was extracted. Relative TLR gene expression levels

were then examined by qRT-PCR and the hprt gene was used as the internal control to calculate ΔCt values. The ΔΔCt values were calculated by subtracting ΔCt values of non-primed samples upon TLR stimulation ('TLR ligand') from ΔCt values of samples treated with aSyn-priming upon TLR stimulation ('Wt+TLR ligand'), to give the fold-change in TLR expression in cells with 'aSyn+TLR ligand' relative to 'TLR ligand' treatments. In all cases the TLR gene analysed (indicated inside bars) corresponded to the TLR agonist used in that particular sample. Fold-changes represent the average of three independent experiments (N=3), each one performed with duplicate samples. Bars correspond to SEM. Statistically significant differences (* $p<0.05$) were calculated by applying the Student t test between the two sets of results, for all the TLR ligands tested. doi: 10.1371/journal.pone.0079160.g002

## Impact of aSyn priming on microglial phagocytic capacity upon TLR stimulation

Recently, it was reported that, in contrast to the aggregated form, monomeric aSyn enhances the microglial phagocytic capacity (Park et al. 2008). Indeed, our previous observation that non-aggregated Wt aSyn promotes a moderate but significant increase in microglial phagocytosis (Roodveldt et al. 2010) is consistent with this finding.

Therefore, we sought to test whether or not the changes previously observed in the secretion of specific cytokines for aSyn-primed microglia, following stimulation by certain TLR ligands, could be accompanied by alterations in the relative phagocytic capacity of microglia that could be possibly linked to the pathogenesis of the synucleinopathies. For this purpose, we used fluorescein-conjugated tracker microparticles to test the phagocytic capacity of aSyn-primed vs. non-primed microglia, following stimulation with certain TLR ligands (**Figure 3**). Despite the fact that a certain trend towards moderate increases in microglial phagocytosis was noticed in the cases of stimulation with (TLR2/1) Pam3 of Wt-primed microglia (~30%), the differences did not reach statistical

significance (**Figure 3**) and therefore we can conclude that the contribution of aSyn preconditioning in the neurodegenerative process or immune imbalance after TLR triggering, within the microglial environment, is independent of phagocytosis.

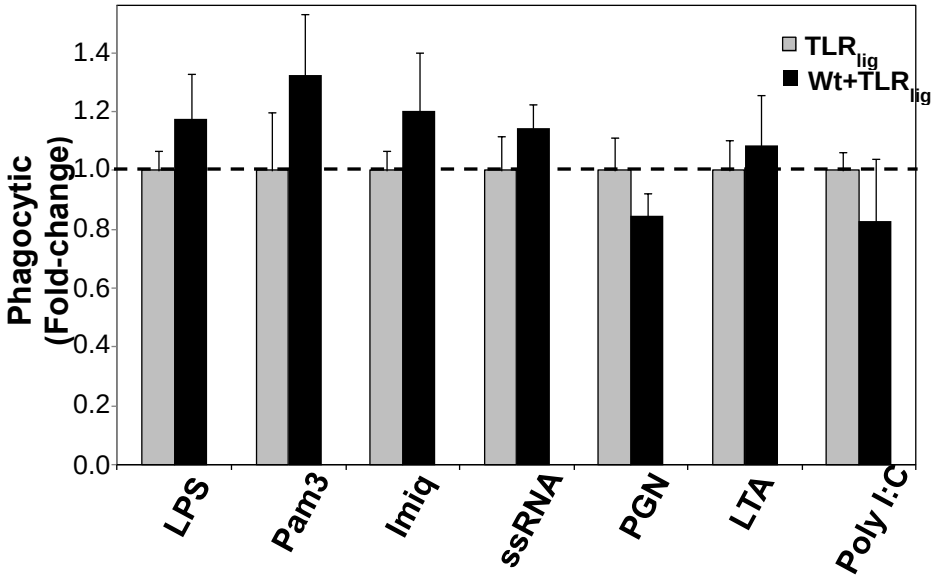



**Figure 3. Relative phagocytic capacity of Wt aSyn-primed vs. non-primed microglia after TLR stimulation.** After treatment of the primary microglial cell cultures and incubation for a total of 24 hrs as described in the legend to Figure 1, cells were incubated with fluorescent microspheres for 1 hr. After fixing the cells, phagocytosis was assessed by fluorescence microscopy and calculation of the number of spheres/cell as described in the Methods section. Four images were analysed for each sample in each independent experiment. The 'relative phagocytic capacity' corresponds to the ratio (fold-change) of the number of spheres/cell of aSyn-primed cells followed by TLR stimulation ('aSyn+TLR ligand') relative to the number of spheres/cell of non-primed, TLR-stimulated cells ('TLR ligand'). The values shown are an average of three (for ssRNA and PGN) or four (for all the others) independent experiments (N=3 or 4), and error bars represent the SEM. Statistically significant differences (* $p<0.05$) were calculated by applying the Student t test between the two sets of results, for all the TLR ligands tested. (#) denotes a result that is significantly different from that obtained after

treatment of cells with Wt αSyn alone ($p<0.05$). doi: 10.1371/journal.pone.0079160.g003

**aSyn preconditioning of microglia followed by stimulation with TLR ligands leads to differential profiles of cell polarization**

In recent years, it has become clear that, as a result of exposure to microenvironmental signals, microglial cells can undergo alternative polarized activation modes. The two extreme phenotypes of macrophages are defined as M1 (the classical, proinflammatory macrophages) and M2 (the 'alternatively activated'/resolving anti-inflammatory cells); however, a full spectrum of activation states which share some overlapping properties with those of the poles, are currently thought to exist (Shechter & Schwartz 2013). To gain further insight into the effect on cell phenotype of aSyn-primed microglia after stimulation withPam3 and ssRNA TLR ligands, we assayed the gene expression of standard M1 and M2 phenotypic markers iNOS and Arg1, respectively (Ponomarev et al. 2013). As can be observed (Figure 4), treatments with either Pam3 or ssRNA alone produced an iNOS+/Arg1- phenotype which, in combination with their cytokine release profiles (higher secreted levels of IL-6 or IP-10 and lower levels IL-10) (**Table 1, Figure 1**), indicate varying degrees of polarization towards an M1-like state.

Interestingly, treatment of aSyn-primed cells with ssRNA (as well as with Wt aSyn alone) produced an iNOS-/Arg1- (double negative) phenotype, while stimulation of cells with A30P aSyn induced the expression of the Arg1 marker. Remarkably, treatment of aSyn-preconditioned microglia with Pam3 agonist, just like exposing cells to oligomeric aSyn, produced an iNOS+/Arg1+ (double positive) intermediate phenotype (**Figure 4**). This result was also the case for treatment with LPS, which is consistent with reports of 'M2 skewing' and the

Arg1+ phenotype of microglia produced by administration of LPS in vitro (Zhang et al. 2009) and in vivo (Chen et al. 2012; Lee et al. 2010). The iNOS+/Arg1+ phenotype observed in these particular samples, together with the increase in secreted IL-6, TNFα, IP-10 and MCP-1 levels (**Tables 1 and 2**) and with an essentially unaltered phagocytic capacity (**Figure 2**), suggests a skewing towards an M1/M2 intermediate or mixed microglial phenotype.

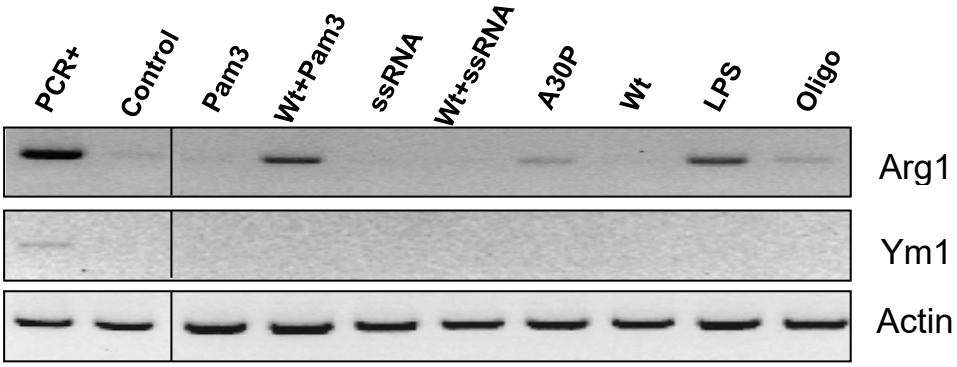
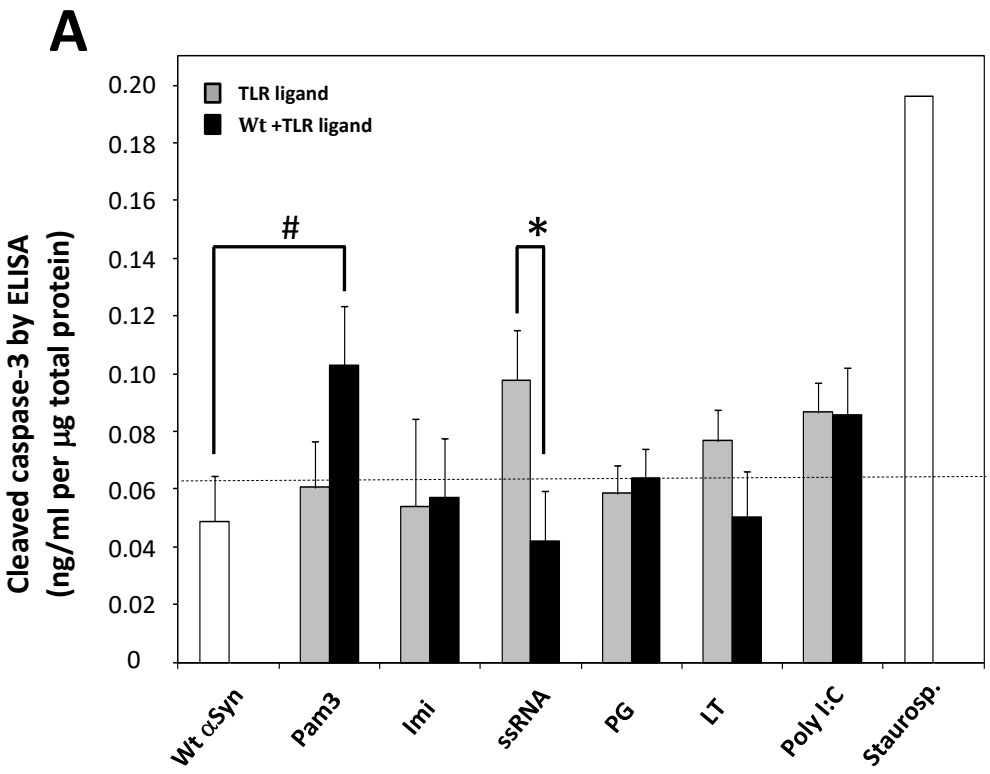



**Figure 4. Arg1 and iNOS PCR gene expression assays of aSyn-preconditioned microglia stimulated with Pam3 or ssRNA**. Primary microglial cells were either treated with Wt aSyn (Wt), A30P aSyn (A30P), or oligomeric Wt aSyn (oligomers) at 1 µg/mL, or with culture medium alone, for 6 hrs at 37 °C. Subsequently, the TLR agonists or medium alone were added accordingly, and cells were incubated for a further 18 hrs as described before. After treatment, RNA was extracted and reverse transcribed for PCR analysis of arginase-1 (Arg1) and iNOS gene expression. Actin expression was used as a reference and the positive controls for the PCR assays (PCR+) were from bone marrow-derived macrophages stimulated (for Arg1) or not (for iNOS) for 24 hrs with IL-4 (10 ng/mL). doi: 10.1371/journal.pone.0079160.g004

**aSyn-preconditioning alters activated caspase-3 levels in microglia following stimulation with Pam3 and ssRNA**

Even though there is a strong link established between the activation of microglia and the progression of PD, the molecular pathways linking microglia-

mediated neuroinflammation and neurodegeneration have been elusive. Activated caspase-3 has been observed in the SN of patients with PD (Hartmann et al. 2000; Tatton 2000; Jellinger 2009), specifically in microglia within the SN of human subjects suffering from PD and AD (Burguillos et al. 2011). Furthermore, caspase-3 was recently shown to have a key role in the regulation of microglia activation and neurotoxicity (Burguillos et al. 2011). Therefore, we sought to compare the expression of activated caspase-3 in primary microglia produced by the different treatments (**Figure 5A**). Except for an increase produced by stimulation of cells with (TLR7) ssRNA and (TLR3) Poly I:C, the basal cleaved caspase-3 levels were not significantly altered by treatment with the TLR ligands or the Wt aSyn alone. However, higher cleaved caspase-3 levels were indeed observed for Wt aSyn-preconditioned microglia upon stimulation with (TLR2/1) Pam3. Remarkably, the observed increase induced by (TLR7) ssRNA alone was strongly suppressed by preconditioning of cells with Wt aSyn (**Figure 5A**). Furthermore, these alterations were also observed by immunofluorescence (IF) analyses of primary microglial cultures that were similarly treated, by using specific antibodies against cleaved caspase-3 (**Figure 5B and Figure 6**). As observed for the untreated cells ('control'), treatment of microglia with Wt aSyn or (TLR2/1) Pam3 alone barely produced detectable levels of activated caspase-3. However, preconditioning of cells with Wt aSyn and subsequent stimulation with Pam3 produced a general increase in fluorescence (ca. 2-fold, $p=0.031$), located primarily in the cytosol (**Figure 6**). Finally, stimulation of cells with (TLR7) ssRNA ligand alone produced an increase in cleaved caspase-3 protein, which localized essentially in the cytosol. In agreement with the results obtained by ELISA (**Figure 5A**), preconditioning of cells with Wt aSyn was found to suppress such an ssRNA-induced increase, by IF analysis. Moreover, this lower level of activated caspase-3 was observed mainly to localize in the cell nuclei (**Figure 6**).


A
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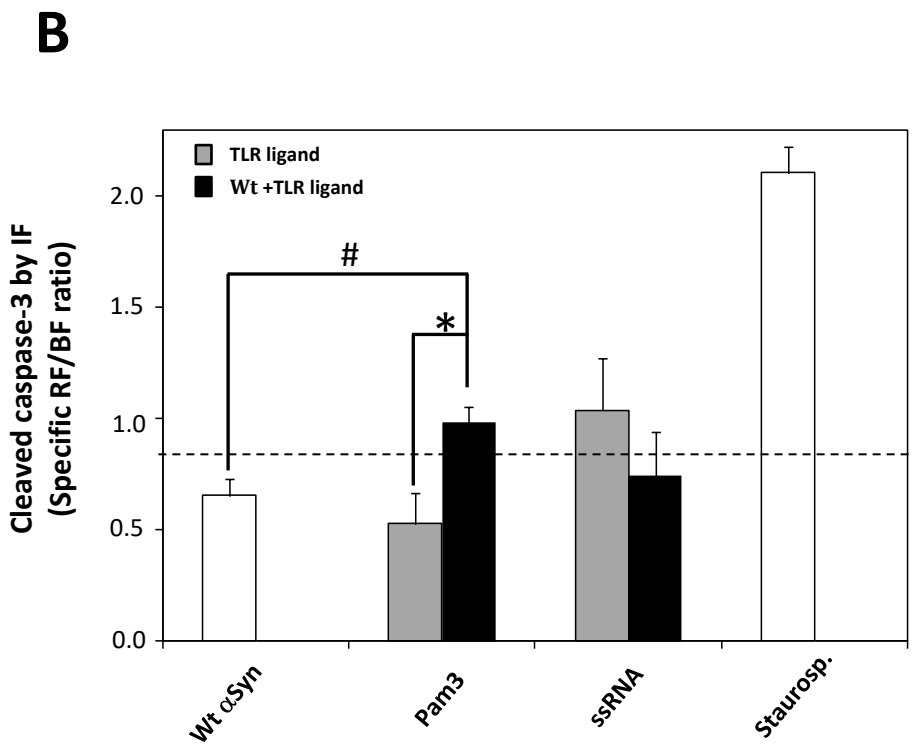
B
TLR ligand
Wt +TLR ligand
Cleaved caspase-3 by IF (Specific RF/BF ratio)
2.0
1.5
1.0
0.5
0.0
#
*
Wt αSyn
Pam3
ssRNA
Staurosp.

**Figure 5. Quantitation of activated caspase-3 levels in treated microglial cells, by ELISA and immunofluorescence**. (A) After treatment of the primary microglial cell cultures and incubation for a total of 24 hrs as described in the legend to Figure 1, cells were lysed and tested by a specific ELISA assay for cleaved caspase-3 levels quantitation. The results shown (ng/mL cleaved casp-3 per µg of total protein) correspond to the mean of four independent experiments (N=4), each one performed with duplicate samples. Bars correspond to SEM. A discontinuous line represents the mean value obtained for untreated cells. Statistically significant differences were calculated by applying the Student's t test in relation to the values obtained with the corresponding TLR ligand in the absence of aSyn-preconditioning (* $p<0.05$) and with Wt aSyn alone (# $p<0.05$). Treatment with staurosporine from Streptomyces sp. (5 µM) for 6 hrs was used as a positive control. (B) Cells were cultured in appropriate culture plates and treated as explained above (see legend to Figure 1) for subsequent labelling of cleaved caspase-3 and nuclear Hoechst 33342 staining for IF analysis, as described in the Methods Section. Samples were analyzed under the fluorescence microscope and three images from random fields containing ca. 80-90 cells each, were recorded, and analyzed for fluorescence quantification. The total specific red fluorescence (RF) and blue fluoresce (BF) were measured and the RF/BF ratio was used as a quantitation method and is represented in this figure. The results shown (RF/BF ratio) correspond to the mean of three images analysed (N=3) within one representative experiment, and bars correspond to SEM. A discontinuous line represents the mean value obtained for images from untreated cells. Statistically significant differences were calculated by applying the Student's t test in relation to the values obtained with the corresponding TLR ligand in the absence of aSyn-preconditioning (* $p<0.05$) and with Wt aSyn alone (# $p<0.05$). Treatment with staurosporine from Streptomyces sp. (5 µM) for 6 hrs was used as a positive control. doi: 10.1371/journal.pone.0079160.g005

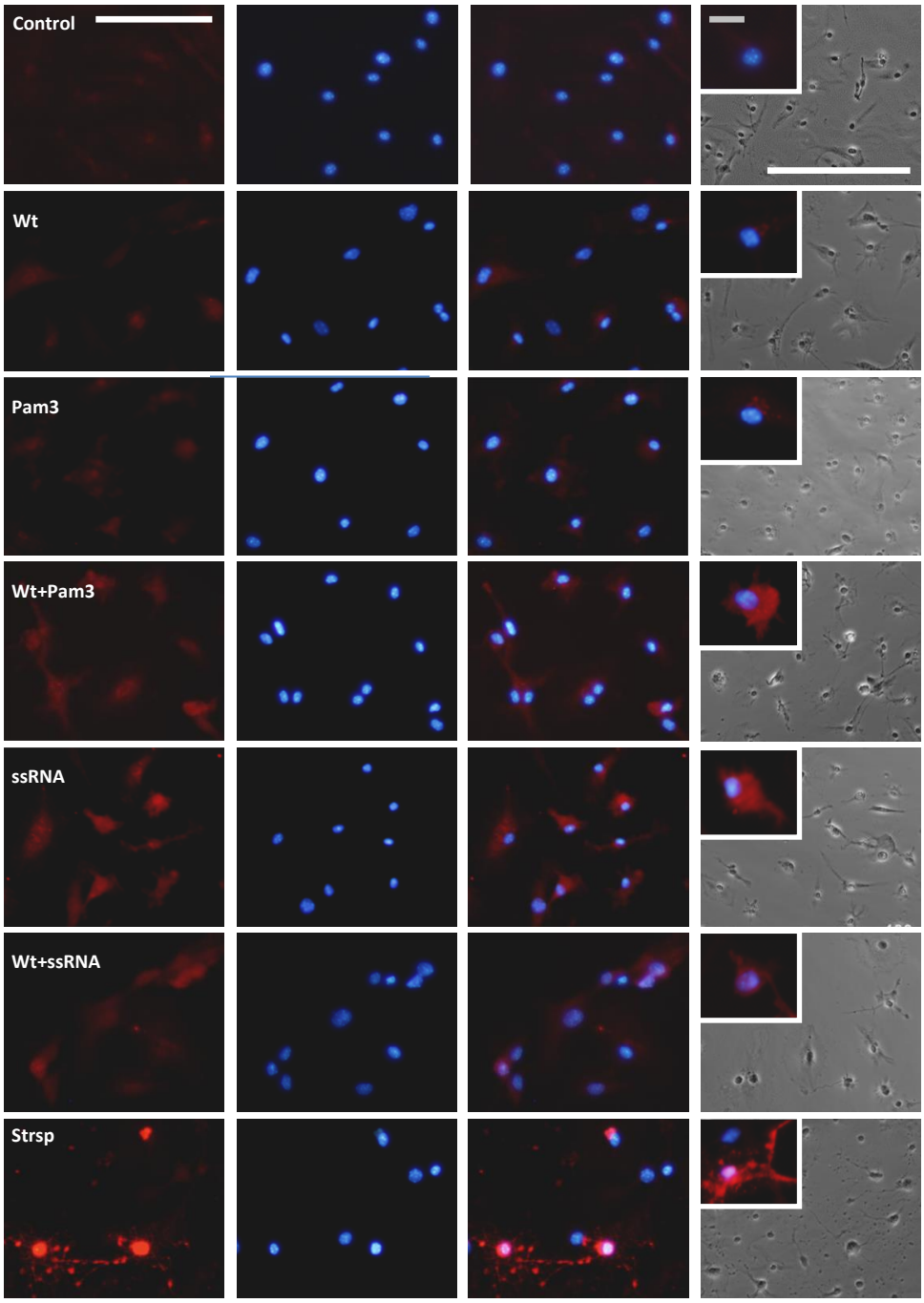


**Figure 6. Immunofluorescence analysis of cleaved caspase-3 levels in treated microglial cells**. Representative images taken from immunolabeled primary microglial cells treated as described in the legend to Figure 1, with or without preconditioning by

Wt αSyn. The TLR ligands tested were Pam3 and ssRNA40 at the concentrations described before (see Materials and Methods). Cells treated with 5 μM staurosporine (Strsp) from Streptomyces sp. for 6 hrs served as a positive control. Specific anti-(Asp175) cleaved caspase-3 primary antibodies and Alexa Fluor 594 secondary antibodies were used to visualize activated caspase-3 (first column), and nuclei were counterstained with Hoescht (second column). Merged images are shown in the third column and phase-contrast images of the same cultures are shown in the right column. White scale bars: 50 μm; grey scale bar (inset): 5 μm.

## 5. Discussion

Despite much progress done in recent years, the underlying mechanisms that trigger the onset of the sporadic form of several neurodegenerative diseases including AD, PD, DLB, and ALS, remain to be elucidated. Given that they all have in common a strong inflammatory response mediated by activated microglia, the existence of additional factors that could potentially exacerbate such neuroinflammatory process is currently thought to be pivotal. Indeed, it has been proposed that microglia in the neurodegenerated brain are somehow 'primed', and signals from systemic infection or inflammation trigger an enhanced response that contributes to disease progression (Perry et al. 2007).

In previous studies, by comparing the effects on primary microglia of Wt aSyn with those produced by the PD-linked aSyn mutants, we and others have observed a strong proinflammatory microglial response for the A30P and E46K variants, as compared to Wt aSyn (Fellner et al. 2011; Roodveldt et al. 2010). Intriguingly, and for reasons still unknown, the levels of brain extracellular aSyn, including its non-aggregated form, have been found to be largely altered in diagnosed patients for several neurodegenerative disorders including AD, PD, DLB, and the prion disease (Mollenhauer et al. 2008; Mollenhauer et al. 2011; Mollenhauer et al. 2013; Tokuda et al. 2006; Tokuda et al. 2010).

In the present work, we have addressed the question of whether or not preconditioning with non-aggregated Wt aSyn could possibly affect the innate immune response of microglia under conditions of TLR challenge. This issue is of great relevance as it may provide information on the microglia-mediated functional innate response upon infection at the very initial stages of disease onset.

Our results show that the impact of aSyn-preconditioning of microglia on the innate immune response following stimulation with TLR ligands largely depends on the nature of the subsequent TLR agonist challenge. Indeed, we observed no significant changes in the cytokine secretion profile for certain TLR ligands tested, including LPS (TLR4). The latter is consistent with previous reports of the response following a challenge with LPS performed both on a transgenic mouse model overexpressing Wt aSyn (Gao et al. 2008) and after injection of non-aggregated Wt aSyn into the mouse SN (Couch et al. 2011), suggesting that similar inflammatory reactions were induced by LPS independently of the presence of αSyn. Interestingly however, we found that Wt aSyn-primed microglia can indeed affect the immune response mediated by TLR2/1 and TLR7, either by increasing the secretion of the pro-inflammatory cytokines IL-6 and TNFα, or by lowering the expression of the anti-inflammatory IL-13. Interestingly, IL-1 has been shown to reduce dopaminergic neuronal cell mortality within a normal environment, but to contribute to their loss under oxidative stress conditions (Morrison et al. 2012).

Very few studies have so far addressed the involvement of chemokines in PD and other related pathologies. In particular, analysis of functional polymorphisms in the genes encoding interleukins and chemokines, and their links with the age of onset or the overall risk of developing PD, has not resulted in any clear associations (Håkansson et al. 2005; Reale et al. 2009). In addition,

the search for chemokine biomarkers of PD in serum has not so far provided useful candidates for diagnosis (Scalzo et al. 2011). In this sense, our results highlight the possible implications of locally affected chemokine environments in primed microglia as a result of specific infections, that have been involved in the recruitment of reactive lymphocytes and in promoting neuronal cell death (Rostène et al. 2016). According to our results, Wt aSyn-priming additionally affects the Pam3 (TLR2/1)- and ssRNA (TLR7) -stimulated microglia by increasing the secretion levels of the chemokines MCP-1/CCL2 and IP-10/CXCL10, respectively, which have been found to be elevated in the CSF of AD brains from the very early stages of disease and to be linked to neurodegeneration (Galimberti et al. 2006). Overall, the change observed in the cytokine release profiles of aSyn-preconditioned microglia stimulated with Pam3 and ssRNA resemble that generated by treatment with aSyn oligomers and with the A30P aSyn variant, both linked to PD and known to elicit a strong inflammatory response mediated by microglia activation (Roodveldt et al. 2010; Zhang et al. 2005). In addition, the differential cytokine secretion profiles upon stimulation with Pam3 and ssRNA are not mediated by significant changes in TLR expression in the case of aSyn-primed cells.

It is now accepted that multiple forms of activated microglia exist, and whether the roles that such differential patterns of activation play in the pathobiology of neurodegenerative diseases are beneficial or detrimental, is currently the subject of much debate (Shechter & Schwartz 2013). Microglial phagocytosis has traditionally been related to steady-state tissue homeostasis by preventing the release of proinflammatory intracellular components from dead or dying cells, and by contributing to the resolution of inflammation (Colton & Wilcock 2010; Schwartz 2010). More recently, microglial phagocytosis has also been shown to have a role on neuronal death during inflammation triggered by TLR

stimulation (Neher et al. 2011). Our present results show that there is no clear effect of aSyn-preconditioning on the phagocytic capacity of TLR-stimulated microglia. In addition, assessment of Arg1 and iNOS gene expression, together with analysis of cytokine release, has revealed a skewing towards an M1/M2 mixed or intermediate activation phenotype for aSyn-preconditioned microglia upon stimulation with ssRNA or Pam3 (**Figure 7**). It is noteworthy that an intermediate M1/M2 microglial phenotype has been found in vivo in AD murine models (Colton & Wilcock 2010). However, the double negative or double positive character of these resulting activation phenotypes suggest that they are of a different nature depending on the particular TLR ligand involved. Our results also indicate that the mixed M1/M2-like response elicited by treatment of aSyn-primed microglia with Pam3 is reminiscent of that displayed after treatment with aSyn oligomers, which are thought to be the most inflammatory and toxic forms of aSyn (Marques & Outeiro 2012; Kalia et al. 2013; Cremades et al. 2012). This finding is highly relevant as it has been demonstrated recently that the inoculation of aggregated forms of Wt aSyn into mouse brains is sufficient to trigger PD-like neurodegeneration and the development of PD characteristic symptoms (Kelvin C Luk et al. 2012; K. C. Luk et al. 2012).

In recent years, a link between activated caspase-3 and PD and AD has been put forward; higher activated caspase-3 levels have been detected in the SN of PD patients (Hartmann et al. 2000; Tatton 2000; Jellinger 2009) and specifically in microglial cells within the SN of PD and AD human subjects (Burguillos et al. 2011). Furthermore, activated caspase-3 was recently shown to play a key role in the regulation of microglia activation and to correlate positively with neurotoxicity, initially as a result of TLR stimulation (Burguillos et al. 2011). In this context, our findings indicate that aSyn-primed microglia result in increased activated caspase-3 levels after TLR2/1 engagement by Gram (+)

bacteria-related Pam3. On the other hand, virus-like ssRNA produces the opposite effect in addition to relocation from the cytosol to the nucleus, which might suggest an activation of the apoptosis pathway (Zheng et al. 1998), and therefore our findings could also be of potential interest for selective manipulation of apoptotic or neurotoxic signalling pathways in a synucleinopathy-prone scenario.

In summary, our results show that extracellular wild-type aSyn could potentially act as a priming factor for microglia to produce an altered TLR response as compared to the same challenge in the absence of such priming. Moreover, we show that the features of this altered response are highly dependent on the identity of the agonist engaging such TLR-mediated responses (**Figure 7**). We propose that this priming effect could be especially relevant in the case of sporadic synucleinopathies and other related disorders with aSyn imbalances since it postulates that specific infections or inflammatory stimuli, even at the pre-oligomeric stage of the aSyn aggregational process, could potentially act as a trigger of an altered microglial response and accelerate the onset of the disease.

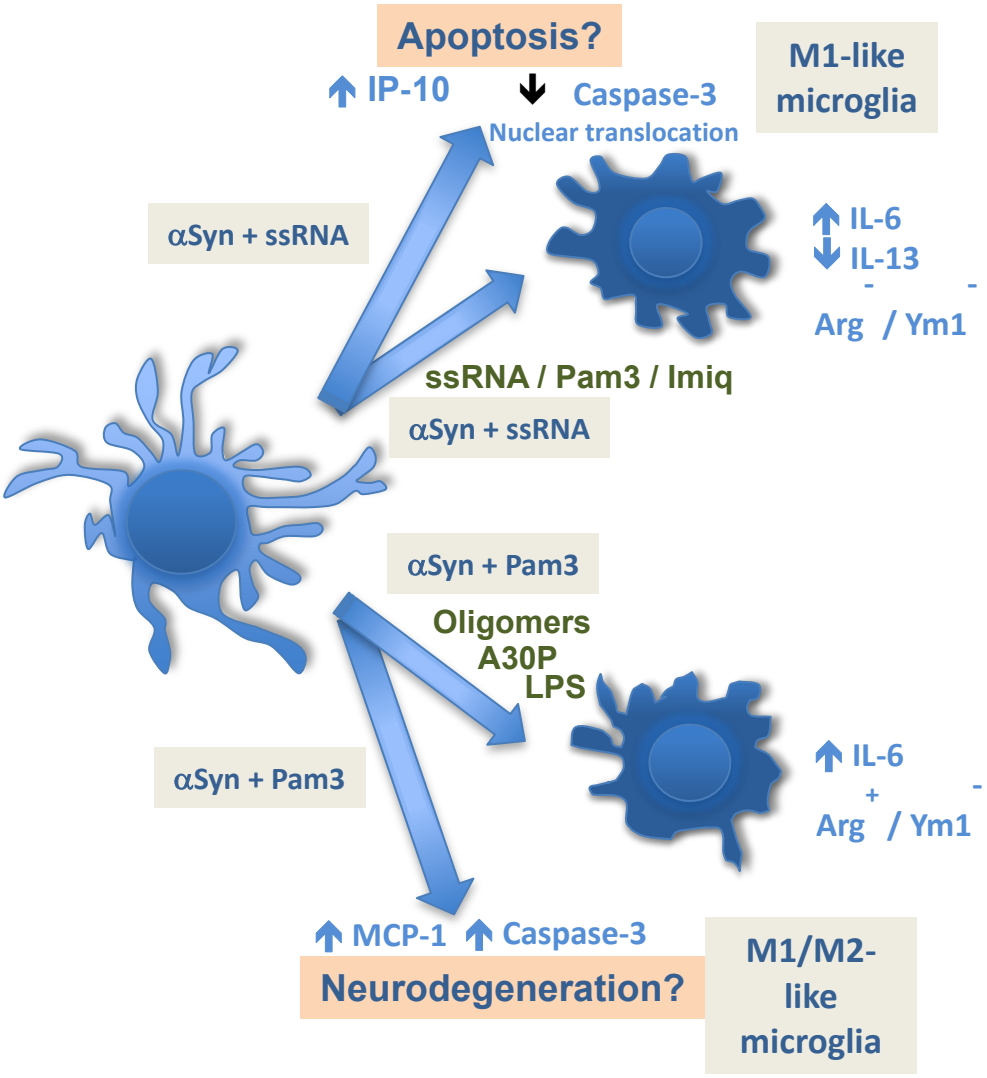


**Figure 7. Proposed model of the impact of αSyn-priming and TLR stimulation on microglial phenotype and neuroinflammation.** Surveyling microglia undergo polarization towards an M1-like phenotype after exposure to (TLR7) ssRNA and Imiq, and (TLR2/1) Pam3, TLR agonists, characterized by a lack of expression of Arg1, expression of iNOS, and high IL-6 production (see Results). On the one hand, αSyn-preconditioning of microglia and subsequent stimulation with (TLR7) ssRNA (and probably Imiq) produces an Arg1-/iNOS- (double negative) mixed or intermediate phenotype, and causes an increase in IP-10 and TNFα secretion, and a reduction of IL-13 levels. In addition, this treatment leads to a reduction in activated caspase-3 levels

accompanied with a change in its intracellular location from the cytosol towards the nucleus of the microglial cell. On the other hand, exposure of αSyn-primed microglia to (TLR2/1) Pam3 agonist induces a skewing towards a different M1/M2 mixed or intermediate phenotype, exhibiting an Arg1+/iNOS+ (double positive) expression pattern, together with higher IL-6 and MCP-1 secretion levels. Remarkably, this phenotype agrees with the one observed for microglia that have been exposed to oligomeric αSyn. In addition, the 'αSyn + Pam3' treatment causes increase in activated caspase-3 levels in microglial cells. doi: 10.1371/journal.pone.0079160.g007

## 6. Acknowledgements

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## 7. Author Contributions

Conceived and designed the experiments: CR DP. Performed the experiments: CR ALG EGR CCL TG RFM ABR GR. Analyzed the data: CR CCL EGR TG AH MD CMD DP. Wrote the manuscript: CR DP.

# CONCLUSIONS

1. Extracellular non-aggregated α-synuclein could potentially act as a priming factor for microglia to produce exacerbated TLR7 and TLR1/2 responses upon further challenge as compared to the same challenge in the absence of such priming.

2. α-synuclein-priming of microglia causes a skewing of the immune response upon stimulation with ssRNA (TLR7) or Pam3 (TLR2/1) TLR ligands, towards M1/M2 intermediate and highly differential phenotypes.

# CHAPTER 2: Immunotherapeutic approximations

Immunity, Inflammation and Disease

Open Access

ORIGINAL RESEARCH

## Chaperoned amyloid proteins for immune manipulation: α-Synuclein/Hsp70 shifts immunity toward a modulatory phenotype

Adahir Labrador-Garrido[1,2], Marta Cejudo-Guillén[1,2], Rebecca Klippstein[1,2], Erwin J. De Genst[3], Laura Tomas-Gallardo[4], María M. Leal[1], Javier Villadiego[5,6,7], Juan J. Toledo-Aral[5,6,7], Christopher M. Dobson[3], David Pozo[1,2], & Cintia Roodveldt[1]

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## Chaperome screening leads to identification of Grp94/Gp96 and FKBP4/52 as modulators of the α-synuclein–elicited immune response

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# OBJECTIVES

During the last decade, it has become accepted that there is a link between α-synuclein and the sustained neuroinflammation and immune imbalance associated to Parkinson's disease. As a result, different approximations have been tested to try to restore it. Molecular chaperones have a key role in preventing abnormal protein aggregation by interacting with misfolding proteins, including α-synuclein. On the other hand, several members of the chaperome network have shown immune modulatory capabilities and to be potentially useful for treating cancer and certain infections. In order to put in common these two features, which are particularly relevant for PD and other neurodegenerative misfolding diseases, we performed a set of experiments to:

1st Characterize the peripheral immune response generated by immunization with the complex-forming Hsp70 and aSyn combination in the absence of added adjuvant. (A.Labrador-Garrido *et al*. *IID* 2014)

2nd Screen a large set of chaperones on their immune-modulatory capabilities to find other promising candidates. (A.Labrador-Garrido *et al*. *FASEBJ* 2015)

3rd Characterize the peripheral immune response elicited by immunization of healthy mice with two selected chaperone candidates, by two different protocols:

3.1 Immunization with Grp94 or FKBP4 in combination with monomeric aSyn (A.Labrador-Garrido *et al*. *FASEBJ* 2015)

3.2 Immunization with Grp94 or FKBP4 in combination with oligomeric aSyn (A.Labrador-Garrido *et al*. *FASEBJ* 2015)

4th Investigate the peripheral immune response and the associated immunity at the CNS level, elicited in a PD mouse model by prophylactic immunization with Grp94 in combination with monomeric aSyn, by two different approximations:

4.1 Adoptive transfer of splenocytes derived from immunized healthy mice to recipient PD model animals (J.Villadiego*, A.Labrador-Garrido* *el al. JNeurosci* 2017 –in preparation-)

4.2 Direct immunization of PD model mice with Grp94 in combination with monomeric aSyn. (J.Villadiego*, A.Labrador-Garrido* *el al. JNeurosci* 2017 –in preparation-)

5th Determine the effect of these two interventions on dopaminergic neuron cell death and on PD symptoms. (J.Villadiego*, A.Labrador-Garrido* *el al. JNeurosci* 2017 –in preparation-)

# Chaperoned amyloid proteins for immune manipulation: a-Synuclein/Hsp70 shifts immunity toward a modulatory phenotype

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## 1. Abstract

a-Synuclein (aSyn) is a 140-residue amyloid-forming protein whose aggregation is linked to Parkinson's disease (PD). It has also been found to play a critical role in the immune imbalance that accompanies disease progression, a characteristic that has prompted the search for an effective aSyn-based immunotherapy. In this study, we have simultaneously exploited two important features of certain heat-shock proteins (HSPs): their classical ''chaperone''

activities and their recently discovered and diverse ‘‘immunoactive’’ properties. In particular, we have explored the immune response elicited by immunization of C57BL/6 mice with an aSyn/Hsp70 protein combination in the absence of added adjuvant. Our results show differential effects for mice immunized with the aSyn/Hsp70 complex, including a restrained aSyn-specific (IgM and IgG) humoral response as well as minimized alterations in the Treg ($CD4^+CD25^+Foxp3^+$) and Teff ($CD4^+Foxp3^-$) cell populations, as opposed to significant changes in mice immunized with aSyn and Hsp70 alone. Furthermore, in vitro-stimulated splenocytes from immunized mice showed the lowest relative response against aSyn challenge for the ‘‘aSyn/Hsp70’’ experimental group as measured by IFN-g and IL-17 secretion, and higher IL-10 levels when stimulated with LPS. Finally, serum levels of Th1-cytokine IFN-g and immunomodulatory IL-10 indicated a unique shift toward an immunomodulatory/immunoprotective phenotype in mice immunized with the aSyn/Hsp70 complex. Overall, we propose the use of functional ‘‘HSP-chaperoned amyloid/aggregating proteins’’ generated with appropriate HSP-substrate protein combinations, such as the aSyn/Hsp70 complex, as a novel strategy for immune-based intervention against synucleinopathies and other amyloid or ‘‘misfolding’’ neurodegenerative disorders.

## 2. Introduction

a-Synuclein (aSyn) is a highly conserved, soluble protein which is abundant in various regions of the brain, and which is currently believed to play a role in modulating synaptic plasticity, neurotransmitter release, and presynaptic vesicle pool size (Abeliovich et al. 2000; Murphy et al. 2000; Cabin et al. 2002). However, the aberrant misfolding and aggregation of this protein and, ultimately, its conversion into insoluble amyloid-like fibrils, are linked to Parkinson’s disease and other synucleinopathies (Marques & Outeiro 2012).

These and other ‘‘misfolding/conformational’’ disorders are characterized by conversion of an initially soluble and functional polypeptide into aggregation intermediates to ultimately produce insoluble aggregates that can be structurally amorphous or fibrillar (Chiti & Dobson 2006). In each particular disorder, unfolding and/or misfolding of specific proteins (for simplicity, herein referred to as ‘‘aggregating proteins’’) are key initial steps in the aberrant aggregation and amyloid formation process (Knowles et al. 2014).

Like many of the peptides and proteins that are involved in the most common misfolding and amyloid diseases – including Alzheimer’s, Huntington’s, type II diabetes, and spongiform encephalopathies – aSyn is an intrinsically disordered protein (IDP) in its free soluble form (Knowles et al. 2014). IDPs are a group of polypeptides that lack significant secondary and tertiary structure, as well as many specific intra-chain interactions (Uversky 2013). The high degree of conformational flexibility in IDPs has been suggested to underlie the observations that these proteins upon immunization tend to produce weak immune responses (Uversky et al. 2005) and that they help certain pathogens escape immune detection by the host (Dunker 2013).

In the last decade, a link has been established between aSyn and the abnormal immunological process that accompanies the onset and progression of synucleinopathies (Roodveldt et al. 2011; Sanchez-Guajardo, Barnum, et al. 2013). In addition to a robust microglia activation and sustained neuroinflammation in the brain, changes in the T cell mediated immunity in the brain and the periphery – including an increased presence of T effector (Teff) memory cells and dysfunctional regulatory T (Treg) cells – are seen during PD progression (Saunders et al. 2012). In the case of the synucleinopathies, several studies performed in animal models have found that $CD4^+$ T cells are critically involved in the neurodegenerative and neuroprotective antagonistic processes

associated to disease (Sanchez-Guajardo, Annibali, et al. 2013; Brochard et al. 2009; Reynolds et al. 2007; Reynolds et al. 2010). Based on this evidence, it has been proposed that identifying modulators that are able to restore the imbalance in the Treg-/Teff-mediated immunity and to induce a regulated, ‘‘neuroprotective’’ immunological environment, might be the key for developing effective immunotherapeutic strategies against neurodegenerative disorders (Reynolds et al. 2007; Ha et al. 2012; Romero-Ramos et al. 2014).

Several studies carried out following the pioneering work by Masliah et al. (Masliah et al. 2005) have explored different types of approaches to immunization with a-synuclein in animal models, which have produced promising albeit mixed results, or with as yet uncharacterized therapeutic efficacy in human subjects (Sanchez-Guajardo, Annibali, et al. 2013; Benner et al. 2008; Masliah et al. 2005; Masliah et al. 2011; Valera & Masliah 2013; Ghochikyan et al. 2014; Mandler et al. 2014). Therefore, the development of novel aSyn-based vaccination strategies for synucleinopathies and related disorders stands as a highly attractive but challenging avenue for research and development.

In addition to the long-established chaperoning functions of heat-shock proteins (HSPs) including binding to, remodelling, and conformational stabilization of, unfolded/ misfolded client polypeptides (Hartl et al. 2011), certain HSPs have been increasingly reported to play diverse roles as modulators of the innate and adaptive immunity (Henderson et al. 2010; Pockley et al. 2008; Quintana & Cohen 2011; Srivastava 2002). Such functions range from promoting antigen cross-presentation and the maturation of dendritic cells, to exerting immunosuppressive signals, or facilitating the activation of lymphocytes and macrophages (De Maio & Vazquez 2013; Tamura et al. 2012; Murshid et al. 2012; Muralidharan & Mandrekar 2013).

Therefore, HSPs possess two highly valuable features that could be simultaneously exploited for aggregating protein-based manipulation of the immune response against misfolding or amyloid disorders, namely, their classical ‘‘chaperone’’ functions combined with their recently discovered ‘‘immunoactive’’ properties. Indeed, this second property has been used to boost the antibody production by immunizing mice with DnaK (bacterial) HSP chemically cross-linked with Ab peptide (Koller et al. 2004) and PrP protein (Koller et al. 2002), and with a 17-aminoacid sequence from Hsp60 conjugated to an Ab epitope (Nemirovsky et al. 2011), that is by utilizing either inactivated HSP proteins or HSP short fragments, as carriers. Still, to the best of our knowledge, no comprehensive characterization of the immune response elicited by an ‘‘HSP-chaperoned aggregating protein’’ complex – that is a complex generated with a ‘‘functional chaperone’’ able to interact productively with the aggregating protein substrate and display its full biological activities – has thus far been reported. In this study we decided to investigate, as a proof-of-concept, the immune response elicited by immunization with a complex-forming HSP-aSyn combination. We took advantage of the acquired knowledge on Hsp70, a highly conserved HSP (Saibil 2013), which has been shown to bind to aSyn aggregation intermediates (Dedmon et al. 2005; Huang et al. 2006; Roodveldt et al. 2009; Luk et al. 2008) and to interact with aSyn monomers (Roodveldt et al. 2009). We report a differential immunological profile as a result of immunizing naive mice with a combination of highly purified human Hsp70 and a-synuclein proteins in the absence of added adjuvant.

## 3. Material and Methods

### a-Synuclein and Hsp70 protein overexpression, purification, and characterization

Human Wt aSyn was over-expressed in Escherichia coli BL21 (DE3) cells using pT7-7 plasmid and purified as described previously (Roodveldt et al. 2010). The purity and monomeric state of the aSyn protein preparation (>95%) were assessed by15% SDS-PAGE, 4–12% native PAGE (Lonza, Basel, Switzerland), and mass spectrometry (not shown), aspreviously described (Roodveldt et al. 2010). Recombinant N-hexa-His-tagged human Hsp70 (HSPA1A), which was previously cloned into the pET28b vector (Novagen, Merk Millipore, Darmstadt, Germany) was overexpressed in E. coli BL21(DE3) (Lucigen, Middleton, WI, USA) and then purified and treated as described previously (Roodveldt et al. 2009). The purity of the Hsp70 preparation (>95%) was assessed by 12% SDS-PAGE. After passing the protein solution through a Amicon Ultra-100 kDa (Merck Millipore Ltd., Carrigtwohill, IRL), the protein was assayed for its endotoxin content by the ToxiSensor Chromogenic LAL Assay Kit (GenScript, Piscataway,USA). The endotoxin levels of the protein preparations were <1 EU/mg protein in all cases. Protein concentrations were determined by means of Micro BCA Reagent Kit (Pierce, Rockford, IL, USA).

### Preparation of the aSyn/Hsp70 complex

In order to favour the formation of the aSyn/Hsp70 complex, the purified aSyn and Hsp70 proteins were pre-incubated at a1:1 molar ratio in ‘‘Hsp70 buffer’’ (50mM Tris/HCl pH 7.4;150mM KCl, 2mM MgCl2) in the presence of 4mMadenosine 50-triphosphate magnesium salt (ATP) (Sigma–Aldrich, St. Louis, USA) for two hours at room temperature(RT), after which time adenosine

50-diphosphate monopotassium salt dehydrate (ADP) (Sigma–Aldrich St. Louis,USA) was added to a 2.5mM final concentration and incubated for a further two hours at RT. Sample preparations consisted of aSyn alone, Hsp70 alone, a mixture of both, orHsp70 buffer, and they all contained the same buffer and received the same incubation treatment. For immunization purposes, samples were diluted accordingly in PBS after incubation.

**Western blot assay for aSyn/Hsp70 complex characterization**

In order to assay the formation of the aSyn/Hsp70 complex, protein preparations were loaded onto a 4–12% native PAGE (Lonza, Basel, Switzerland) and subjected to electrophoresis at 120 V, and transferred for 45 min onto 0.2mm nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK).After blocking overnight with 5% skimmed milk in PBST (0.05% Tween 20 in PBS), the membranes were probed with the mouse anti-a/b-synuclein (N19) polyclonal antibody(Santa Cruz Biotechnology Inc. Heidelberg, Germany) or the anti-Hsp70 monoclonal antibody (C96F3-3) (Enzo LifeSciences inc. Farmingdale, NY, USA). HRP-conjugated anti-goat (Santa Cruz Biotechnology inc. Heidelberg, Germany)and anti-mouse (Promega, Madison, WI, USA), secondary antibodies were used to visualize blots by using Immobilion$^{TM}$ Western Chemiluminescent HRP Substrate (Millipore,Billerica, MA, USA) and Amersham HyperfilmTM ECL(GE Healthcare, Buckinghamshire, UK).

**Surface plasmon resonance detection of a-synuclein-Hsp70 interaction**

Surface plasmon resonance experiments were performed in a Biacore X100 instrument with a CM5 sensor chip (GE Healthcare). 50nM Hsp70 (ligand) was immobilized through the amine coupling chemistry, as follows. Both flow cells were activated for 7 min with a 1:1 mixture of 0.1MN-hydroxysuccinimide

(NHS) and 0.4M 3-N,N-dimethylamino(EDC) at a flow rate of 5mL/min. Immobilization was performed in sodium acetate buffer (pH 5.0). The ligand (originally in a solution in ‘‘Hsp70 buffer’’) was injected at 5mL/min in 10mM sodium acetate buffer (pH 5.0) at a concentration of 480nM on the activated sensor surface2 (Fc2) and then 1Methanolamine-HCl (pH 8.5) was added to block the unreacted N-hydroxysuccinimide groups. The level of immobilized ligand was 3173 response units (RU).The sensor surface 1 (Fc1) was activated/deactivated without ligand for its use as reference surface for subtraction of nonspecific signal effects. The running buffer for all the experiments was 1x PBS supplemented with 0.05%Surfactant P20, 2mM MgCl2, and 2.5mM adenosine 50-triphosphatedisodium salt (ATP). All samples were diluted in running buffer at 0, 20, 40, 80, 160, and 320mM, in increasing concentrations order with at least one duplicate of lower concentration after the highest analyte concentration. Contact time for binding and dissociation time were 300 sec. All the binding cycles were performed at 158C. No regeneration was applied to the sensor surfaces as sensorgrams readily returned to the baseline in the dissociation phase. An extra wash with running buffer was applied at the end of each cycle.

Interaction data were analysed using the Biacore X100Evaluation Software (GE Healthcare). The equilibrium dissociation constant (KD) was calculated from steady-state sections of the curves 5 sec before analyte injection stop, by using the Affinity Wizard tool.

**Animals**

Six to seven week old, C57BL/6 male mice were purchased from the University of Seville Center for Animal Production and Experimentation (Espartinas, Spain). Animals were kept for one week in the local animal house before the

start of the immunization protocol, to allow the mice to acclimatize to their new environment. At all stages of the study, animals from each experimental group were allocated into different cages such that each cage contained up to five mice, in every case corresponding to a mix from different experimental groups. All animal procedures were in accordance with good animal practice as defined by the relevant national/EU and ARRIVE guidelines and the CEEA-CABIMER Experimental Animal Committee, and all animal procedures were approved by the corresponding committee (CEEA-2010-14).

**Immunization protocol**

Mice were immunized on day 0 with 5 or 50 μg of Hsp70 (''low'' or ''high'' dose, respectively) and/or 1.06 or 10.6 μg of aSyn (''low'' or ''high'' dose, respectively). All preparations were diluted in PBS (ca. 50-fold dilution of the preincubation mixtures) in the absence of added adjuvants. Mice were injected with a single 100 mL s.c. shot in the lumbar region. The same procedure was repeated on day 7. On day 14, mice were sacrificed, and the spleen and 500–700 mL of blood, were extracted for analyses.

**Determination of $CD4^+$, Treg and Teff cell populations**

Splenocytes were isolated from the spleen of immunized mice by perfusing with 10 mL with PBS after which erythrocytes were lysed by osmotic shock. The number of cells was determined by counting them in a hemocytometer and 106 cells were labelled with anti-CD4-FITC, anti-CD25- APC, and anti-Foxp3-PE, antibodies (BD Biosciences, San Diego, CA, USA), by following the manufacturer's instructions. Flow cytometry analysis was performed with FACS Calibur cytometer and CellQuest Pro (BD Biosciences, San Diego, CA, USA) software.

The Treg cell population was calculated as the percentage of cells positive for CD4, CD25, and Foxp3 staining among the $CD4^+$ lymphocyte population. The Teff (non-regulatory) cell population was calculated as the percentage of cells that stained positively for CD4 and negatively for Foxp3 from the $CD4^+$ lymphocyte population (Supporting Information Fig. S1).

**In vitro stimulation of splenocytes and determination of secreted cytokines**

Splenocytes were isolated from the spleen of immunized mice after sacrifice, as previously described. 3#106 cells from each mouse were divided and cultured in three wells (of a 12-well plate) in RPMI medium (BioWhittaker, Verviers, Belgium) with 10% inactivated foetal bovine serum (FBS, BioWhittaker, Verviers, Belgium). Each well was treated as follows: well 1, medium alone (control); well 2, aSyn (20µg/ml); well 3, lipopolysaccharide from Escherichia coli serotype 0127:B8 (LPS) (Sigma–Aldrich, St. Louis, USA) (0.5mg/mL). After incubation for 24 h, supernatants were collected and centrifuged at 500 g for 5 min to eliminate any remaining cells and debris, and stored at -80°C for subsequent cytokine assaying.

For quantifying IFN-g, IL-10, and IL-17 levels from culture supernatants, specific ELISA kits, namely Mouse IFN-gamma and Mouse IL-10 BD OptEIATM kits (BD Biosciences, San Diego, CA, USA), and ELISA Development Kit Murine IL17 (PreproTech, London, UK), were used according to the manufacturer's instructions.

**Antibody content and cytokine measurement in mouse sera**

Blood samples extracted after sacrifice were left for 1 h at 48C and 1h at RT to let them clot. After clot formation samples were centrifuged at 21,000 g for 15 min to obtain cell-free serum, and stored at "808C for further analyses.

To assay the content of total IgM and IgG antibodies, samples were diluted 1:240,000 in PBS and 100 mL aliquots were transferred to a 96-well plate well (MaxiSorp plate, NUNC, Roskilde, Denmark) and incubated for 1 h at 378C. Next, wells were washed three times with 350 mL of PBST (PBS, 0.05% Tween20) and blocked for 1 h with Assay Diluent (BD Biosciences, San Diego, CA, USA). After washing as in the previous step, wells were incubated for 1 h at 378C with anti-IgM-(Miltenyi, Bergisch Gladbach, Germany) or anti-IgG-(Promega, Madison, WI, USA) -HRP conjugated secondary antibodies diluted 1:4000 in Assay Diluent (BD Biosciences, San Diego, CA, USA). Afterwards, wells were washed five times with PBST, and 3,30,5,50-tetramethylbenzidine (TMB) substrate reagent was added to determine the antibody content by following the manufacturer's instructions. To measure the anti-aSyn specific antibodies in the serum samples, a similar protocol was followed but with some modifications. MaxiSorp (NUNC, Roskilde, Denmark) plates were coated with 1mg of aSyn diluted in 100mL of PBS in each well and incubated for 1 h at 378C. After coating, washing, and blocking steps were performed as described above, and 1:40 diluted serum samples in Assay Diluent (BD Biosciences, San Diego, CA, USA) were added and incubated for 2 h at 378C. Detection of IgM and IgG antibodies was performed as in the protocol described above. All samples were analysed in duplicate.

To determine the IFN-g and IL-10 levels, specific ELISA kits, namely Mouse IFN-gamma and Mouse IL-10 BD OptEIA$^{TM}$ kits (BD Biosciences, San Diego, CA, USA), were used according to the manufacturer's instructions.

**Statistical analyses**

Statistical analyses were performed by using the IBM SPSS Statistics 20 pack. For all parameters (T cell populations, antibody determinations, and cytokine measurements), the Kruskal–Wallis one-way analysis of variance was firstly performed to evaluate the existence of significant differences among the experimental groups. In order to determine the differences between groups and to obtain the P values, the non-parametric Mann–Whitney U test for two independent samples was performed. Each group consisted of 5–7 mice (n=5–7). Statistically significant differences were those with $P<0.05$.

## 4. Results

**Preparation and characterization of a monomeric aSyn/Hsp70 complex**

Based on our previous finding that recombinant human Hsp70 chaperone interacts with monomeric aSyn in vitro (Roodveldt et al. 2009), we chose human Hsp70 to evaluate our immunization strategy with aSyn. In the preparation of the aSyn/Hsp70 complex to be used in biochemical studies as well as for immunization protocols, conditions favouring Hsp70/substrate-binding and therefore, the formation of the aSyn/Hsp70 complex, were chosen and applied for all sample preparations (i.e., aSyn/Hsp70 mixture, aSyn and Hsp70 proteins alone, and buffer/vehicle). For this, highly purified aSyn and Hsp70 in buffer (50mM Tris pH 7.4, 150mM KCl, 2mM $MgCl_2$) at a 1:1 molar ratio (or the corresponding protein amount for samples with aSyn or Hsp70

alone) were incubated at RT in the presence of 4mM ATP for 2 h to induce the opening of the Hsp70 substrate binding pocket, and for a further 2 h with ADP added to a final concentration of 2.5mM to favor the formation of the ‘‘high affinity’’ Hsp70/substrate complex (Saibil 2013).

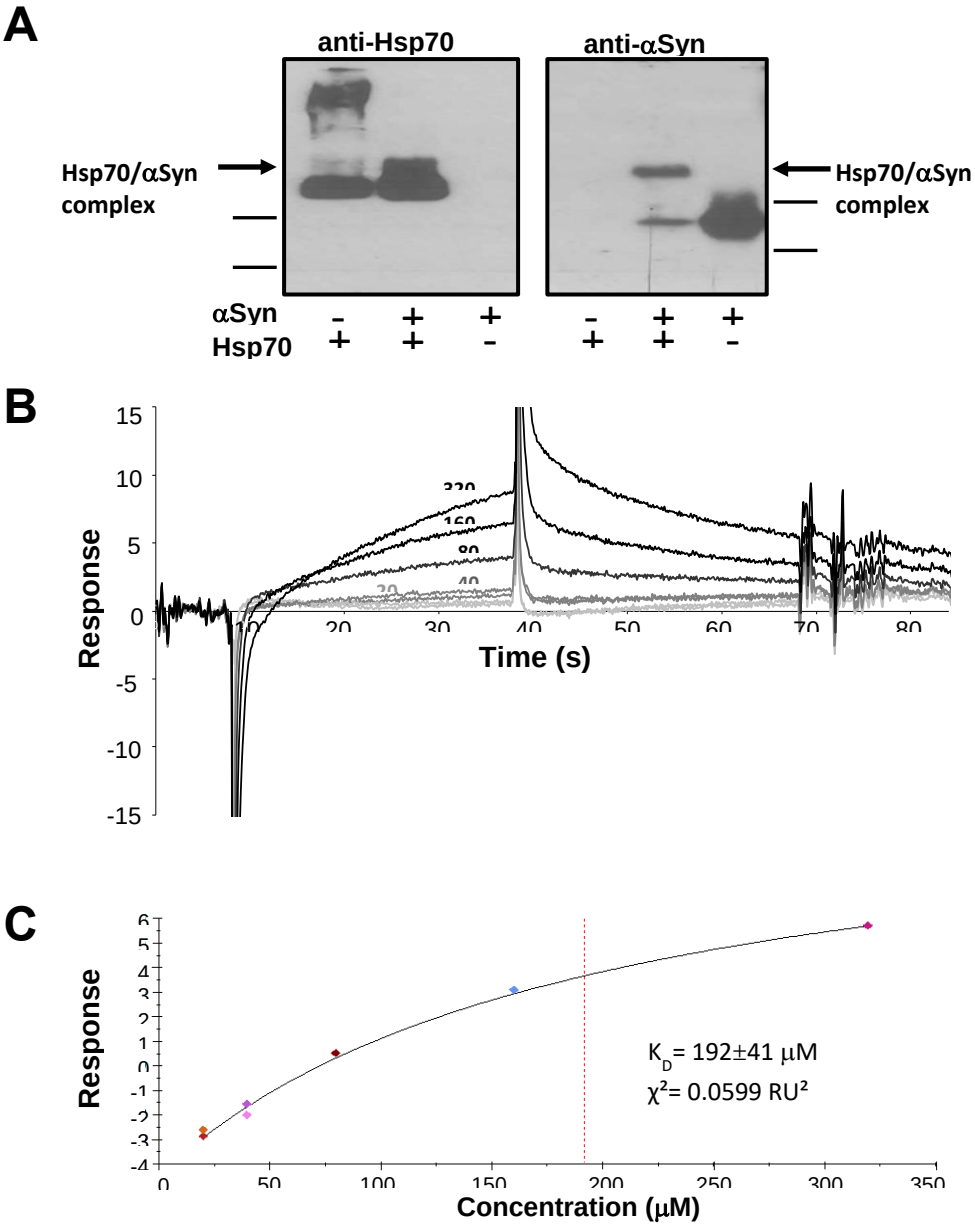


**Figure 1**. Characterization of the Hsp70-monomeric aSyn association and complex formation. Native PAGE and Western blot analysis of samples, by using anti-aSyn or anti-Hsp70 antibodies. In addition to the bands corresponding to Hsp70 and aSyn proteins, a highly overlapping, lower-mobility band can be seen in both Western blots, indicating the formation of an Hsp70/aSyn complex. Bovine serum albumin was used as a protein marker (indicated) (A). Binding of monomeric a-Syn to Hsp70 immobilized on a Biacore sensor chip. Sensorgrams illustrating dosage-dependent binding of a-Syn to 1173 RU of immobilized Hsp70 are represented (B). Steady-state concentration plot of a-Syn bound to Hsp70 5 seconds before the end of the analyte (a-syn) injection (C).

In order to evaluate the formation of an aSyn/Hsp70 complex, we subjected the incubated mixtures to native PAGE electrophoresis followed by Western blot with anti- Hsp70 and anti-aSyn specific antibodies (**Fig. 1A**). Indeed, we were able to detect a band shift in the lane corresponding to the aSyn/Hsp70 sample, as compared to the lanes loaded either with aSyn or with Hsp70 alone in both membranes labelled with either specific antibodies (**Fig. 1A**). This additional band, with apparently similar electrophoretic migration distance in both labelling assays, should correspond to a monomeric aSyn/Hsp70 complex.

To prove the interaction between monomeric aSyn and Hsp70 and further demonstrate the formation of an Hsp70/aSyn complex, we used surface plasmon resonance (Biacore, GE Healthcare). This allowed to monitor the interaction, in real time, between ATP-loaded Hsp70 and monomeric a-Syn through the covalent immobilization of the chaperone onto flow cell 2 (active cell) of the sensor chip and the injection of different a-Syn concentrations through both active and reference, flow cells. Signals from the active cell were corrected by subtracting signal from the reference one. Our data showed that the immobilized ligand (Hsp70) was able to specifically bind to the flowing analyte (monomeric aSyn) in an ATP-containing buffer (**Fig. 1B**). None of the sensorgrams could be fitted to a simple 1:1 Langmuir binding model in order to

calculate kinetic parameters ($K_a$ and $K_d$), probably because the interaction between both molecules is more complex. Binding levels corresponding to a section of the curves 5 sec before the end of the injections were used to calculate the dissociation equilibrium constant ($K_D$). The steady state binding levels against analyte concentration were plotted and fitted to a simple 1:1 fitting model available in the Biacore Evaluation Software. A good fit was obtained ($\chi^2$ value of 0.0599 $RU^2$), with a calculated KD value of 192±41 µM (Fig. 1C), which should correspond to the (ATP) Hsp70/aSyn complex, that is in the ''low affinity'' state (Mayer 2013). Therefore, it can be inferred that the $K_D$ value of the (ADP) Hsp70/aSyn complex induced in our sample preparation for immunization, is lower than 190 µM.

**Restrained levels of anti-aSyn antibodies by aSyn/Hsp70-immunization**

To determine the ability of Hsp70 to act as a paradigm ''chaperone adjuvant'' with aSyn, we designed and tested two immunization protocols in mouse with a combination of human aSyn and Hsp70 leading to the formation of a moderate-affinity complex. For this purpose, highly purified, endotoxin-free recombinant Hsp70 and aSyn proteins were used. Six to seven weeks old C57BL/6 male mice were injected subcutaneously at days 0 and 7, with either ''low'' (5 µg Hsp70 and/or 1.06 µg aSyn) or ''high'' (50 µg Hsp70 and/or 10.6 µg aSyn) protein doses, or buffer (vehicle), and maintaining a ca. 1:1 molar ratio for the mixture between aSyn and Hsp70. One week after the booster injection, mice were sacrificed and their spleen and whole blood were extracted for further analyses.

In order to evaluate the aSyn-specific humoral response resulting from the immunization protocol, we measured the levels of anti-aSyn as well as total IgM and IgG antibodies in serum (**Fig. 2**). For IgM, an increase in both the absolute

anti-aSyn and relative anti-aSyn (calculated as anti-aSyn IgM/total IgM) antibodies was detected in the mouse groups immunized with both doses of aSyn (ca. 35–50%), and with ''low'' Hsp70 (ca. 40%, for absolute IgM levels),

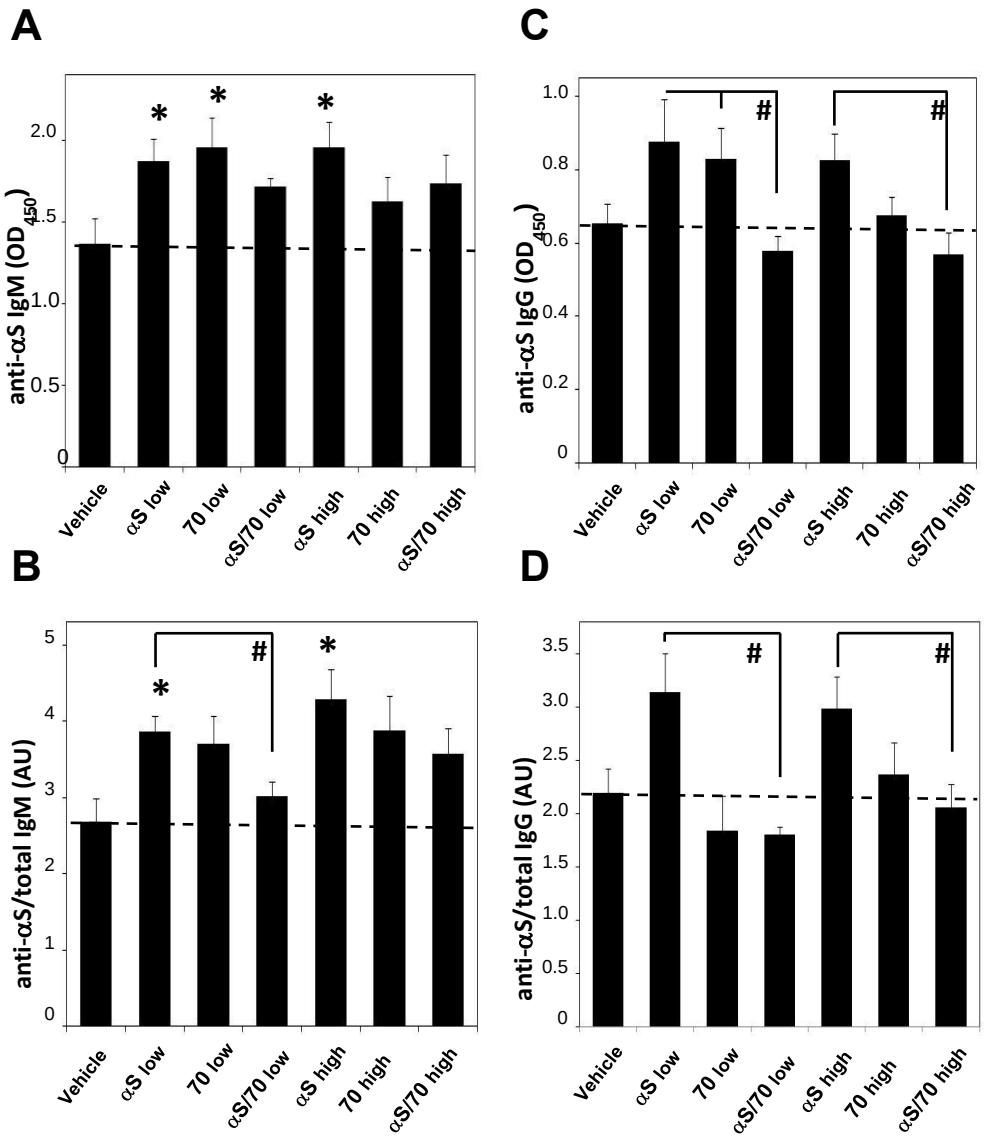



**Figure 2**. Humoral immune response characterization. Absolute anti-aSyn IgM (A) and relative anti-aSyn IgM (B) antibodies in serum from immunized mice. Absolute anti-aSyn IgG (C) and relative anti-aSyn IgG (D) antibodies in serum. The relative content of

specific anti aSyn IgM and IgG antibodies were calculated by dividing anti aSyn IgM or IgG levels by the total corresponding antibody levels. AU: arbitrary units. Represented values are mean±S.E.M. (n=5–7). Asterisks correspond to statistically significant differences between one particular group and the "vehicle" group. Hash signs indicate statistically significant differences between different groups. */# P<0.05, **/## P<0.01.

while this increase was virtually suppressed in the mouse group immunized with the aSyn/Hsp70 combination (**Fig. 2A, B**). A similar profile was observed for the measured IgG antibodies, with similar trends that did not reach statistical significance in absolute anti-aSyn and relative anti-aSyn (calculated as anti-aSyn IgG/total IgG) antibodies, for the ''aSyn'' experimental group (**Fig. 2C, D**). Also in this case, the anti-aSyn IgG antibody levels for both ''aSyn/Hsp70'' mouse groups were close to those in control mice, indicating a differential ''restrain'' effect arising from immunization with the aSyn/Hsp70 combination (**Fig. 2B, D**).

**The regulatory (Treg) and effector (Teff) T cell contents are affected by immunization**

Because of the pivotal participation of $CD4^+$ T cells in the development of synucleinopathies and other misfolding neurodegenerative diseases associated to chronic inflammation, we firstly compared the impact of immunizing mice with vehicle, aSyn, Hsp70, or the aSyn/Hsp70 combination, on the $CD4^+$ cell populations within total splenocytes (**Supporting Information Fig. S1**). Splenocytes were isolated from immunized and sacrificed mice. The content of $CD4^+$ cells in total splenocytes as analysed by flow cytometry showed no significant differences between mouse groups (data not shown).

Next, in order to compare the regulatory T (Treg) cell population contents between the different mouse groups, we quantitated the $CD4^+$ $CD25^+$ $Foxp3^+$-

labeled cells in the isolated splenocytes (**Fig. 3A and Supporting Information Fig. S1**). Our results showed that at ''low'' concentrations of immunogen, both the groups immunized with aSyn and, to a lower degree, Hsp70, produced a significant increase in the Treg percentage (15.7±0.5% and 14.3±0.5%, respectively), as compared to the mouse group immunized with vehicle (control) (11.1±1.0%). However, this effect was not observed using the aSyn/Hsp70 mixture (13.1±1.2%), further indicating a differential effect of this combination (**Fig. 3A**). The mice immunized with high protein doses, on the other hand, did not show any differences in the percentages of Treg cells compared to the control mice.

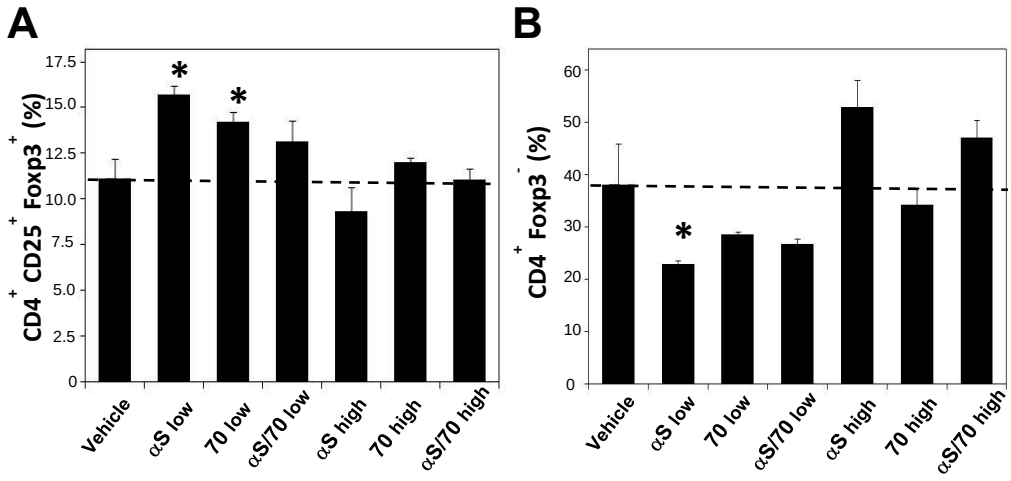



**Figure 3**. Treg and Teff cell populations determination. Percentage of Treg lymphocytes calculated as the percentage of cells positive for CD4, CD25, and Foxp3 staining among the $CD4^+$ lymphocyte population ($CD4^+CD25^+Foxp3^+$) (A). Percentage of Teff lymphocytes calculated as the percentage of cells with positive staining for CD4 and negative for Foxp3 among the $CD4^+$ lymphocyte population ($CD4^+Foxp3^-$) (B). Represented values are mean±S.E.M. (n=5–7). Asterisks correspond to statistically significant differences between one particular group and the "vehicle" group. * $P<0.05$, ** $P<0.01$.

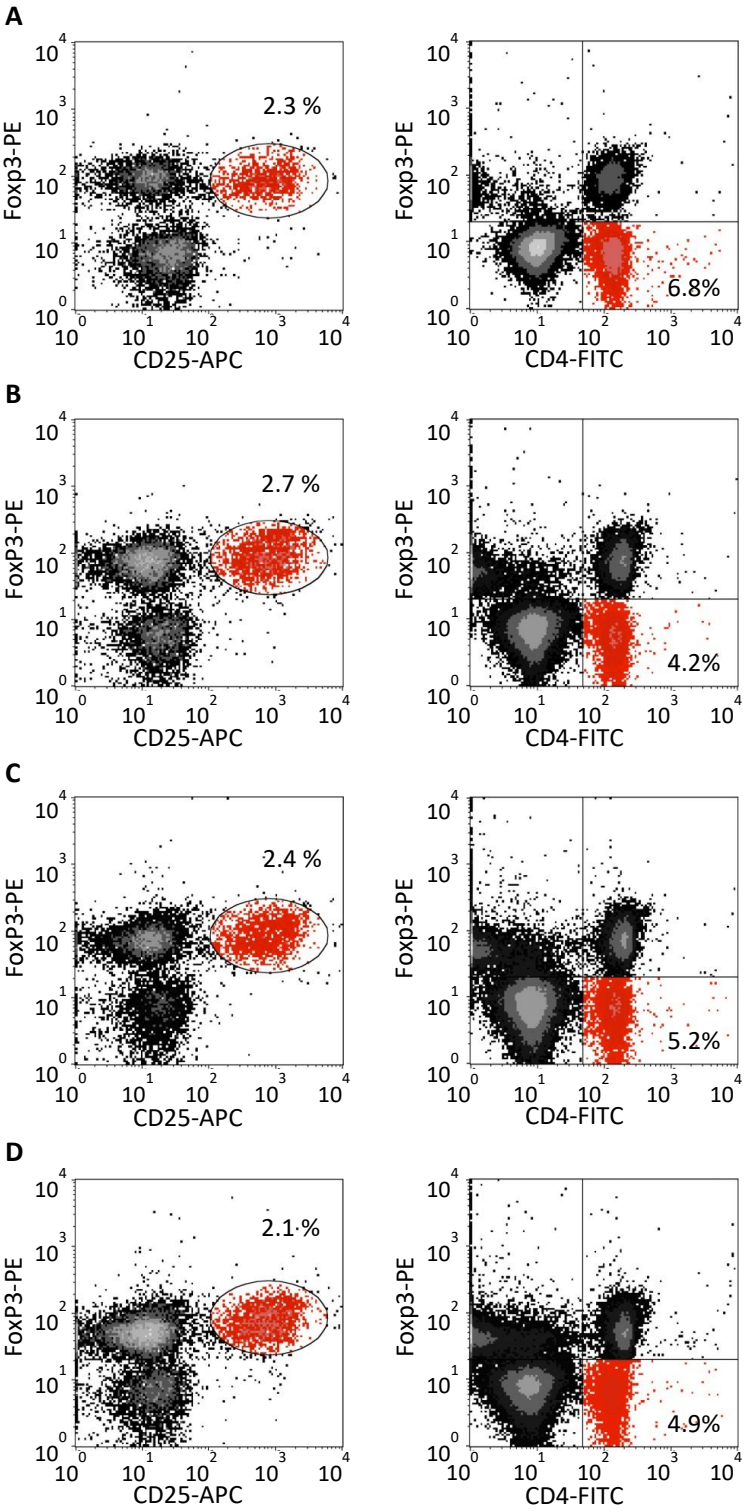

A
2.3 %
Foxp3-PE
CD25-APC
6.8%
CD4-FITC
B
2.7 %
FoxP3-PE
CD25-APC
4.2%
CD4-FITC
C
2.4 %
FoxP3-PE
CD25-APC
5.2%
CD4-FITC
D
2.1 %
FoxP3-PE
CD25-APC
4.9%
CD4-FITC

**Figure supplementary 1**. Representative flow cytometry plots for Treg and Teff populations. The Treg cell population (left column) was calculated as the CD4, CD25 and Foxp3 positive staining among the total lymphocyte population ($CD4^+CD25^+Foxp3^+$). The Teff cell population (right column) was calculated as the percentage of CD4 positively stained cells with negative staining for Foxp3 among the total lymphocyte population ($CD4^+Foxp3^-$). Plots are from one representative mouse of the 'vehicle' (A), 'low' aSyn (B), 'low' Hsp70 (C) and 'low' aSyn/Hsp70 complex (D) groups.

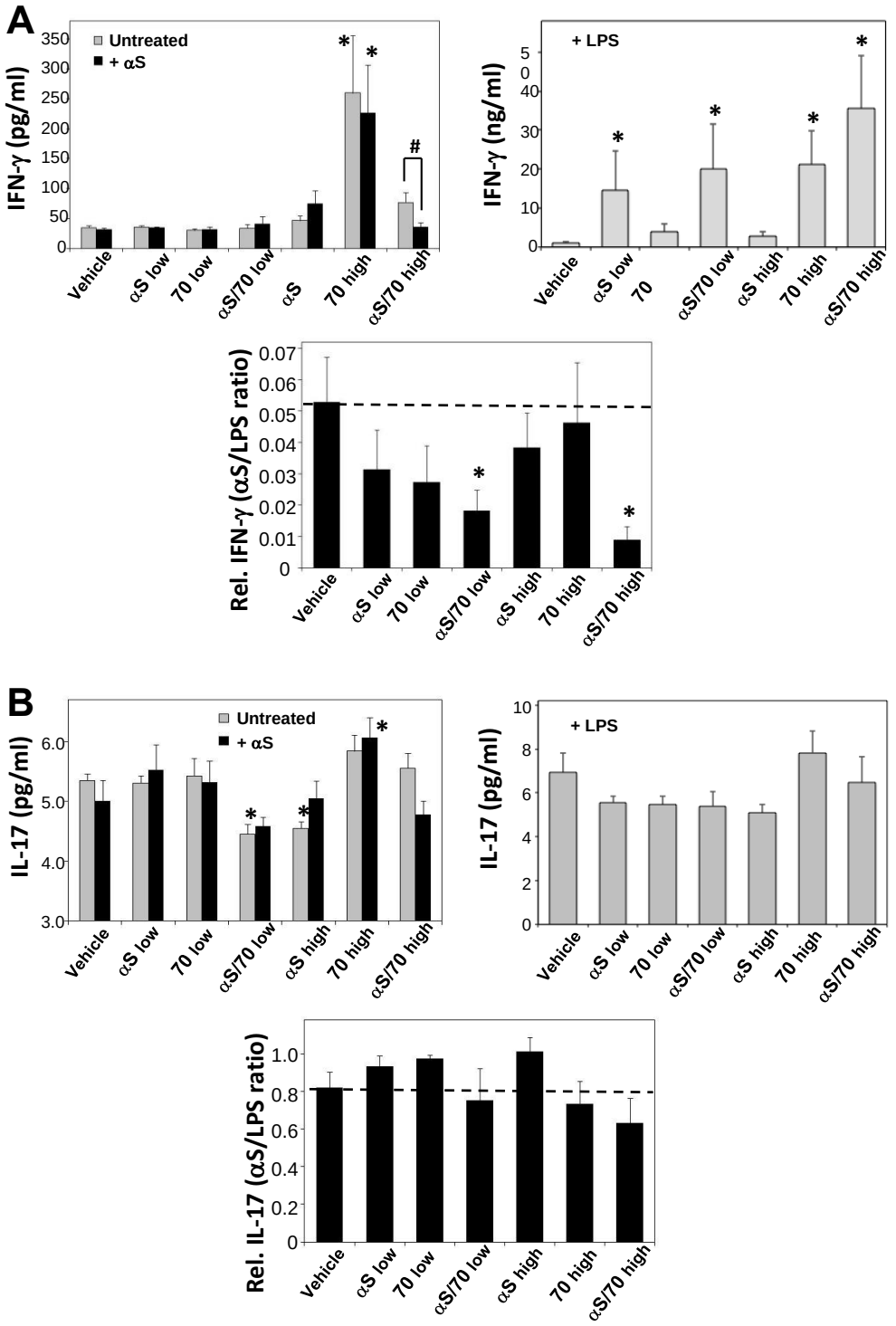
Finally, in order to compare the content of the non-regulatory/effector T (Teff) cell population in the isolated splenocytes from the different mouse groups, we assayed the percentage of $Foxp3^-$ (**Fig. 3B and Supporting Information Fig. S1**). In this case, a significant reduction was measured in the $CD4^+$ $Foxp3^-$ cell population content for the mouse group immunized with a ''low'' dose of aSyn (22.8±0.7%) relative to the group injected with ''vehicle'' (38.1±7.8%) (**Fig. 3B**).

**Differential immunoreactivity of aSyn- versus LPS-pulsed splenocytes from immunized mice**

Next, as a way to monitor possible aSyn-specific, hyper-/hypo-immune responses, we measured cytokine release profiles of cultured splenocytes isolated from immunized mice after sacrifice. To this end the cultured cells from the different experimental groups were pulsed either with 20 mg/mL aSyn (to model the antigen specific immunoreactivity in vitro) or 1 mg/mL LPS (to model non-specific/ polyclonal immunoreactivity in vitro) or, alternatively, medium alone to determine the basal secretion levels, which corresponds to a non-stimulated control (**Fig. 4**). In particular, IFN-γ and IL-17 as well as IL-10, were measured in the supernatant collected after 24 h of incubation. Our results show that splenocytes from mice immunized with a ''high'' dose of Hsp70 produced higher basal levels of IFN-γ (260±97 pg/mL), as compared to splenocytes from mice immunized with vehicle (**Fig. 4A, left panel**). On the

other hand, stimulation of cultured splenocytes with LPS induced higher IFN-γ secreted levels in cells from mice immunized with ''low aSyn'' (15±10 ng/mL) and the ''low aSyn/Hsp70'' complex (20±12 ng/mL), as well as with ''high Hsp70'' (21±9 ng/mL), as compared to the group immunized with vehicle (Fig. 4A, central panel). However, the largest induction of IFN-γ was observed for cells isolated from mice immunized with the ''high aSyn/Hsp70'' combination (36±14 ng/mL) (**Fig. 4A, central panel**). Notably, this higher general reactivity in the case of the high dose of ''aSyn/Hsp70'' did not correlate with that elicited by aSyn pulsing, as lower than basal levels of IFN-γ were measured as a result of aSyn-pulsing of splenocytes for this group (Fig. 4A, left panel). Interestingly, lower values of secreted IL-17 levels for the ''low aSyn/Hsp70'' (4.5±0.17 pg/mL) and ''high aSyn'' (4.6±0.11 pg/mL) groups were measured as compared to the group immunized with vehicle (5.4±0.11 pg/mL), while an increase for the ''high Hsp70'' group (6.1±0.34 pg/mL) relative to the control (5.0±0.35 pg/mL was detected upon aSyn-pulsing of splenocytes (**Fig. 4B, left panel**). On the other hand, no significant differences in secreted IL-17 levels as a result of LPS-stimulation of splenocytes were detected (**Fig. 4B, central, panel**).

Regarding the immunomodulatory-linked cytokine IL-10, splenocytes from all groups, except for the ''low Hsp70'' group, showed higher basal levels of IL-10 as compared to splenocytes from mice immunized with vehicle (three- to fivefold higher) (**Fig. 4C, left panel**). Pulsing splenocytes with aSyn did not significantly alter this profile, except for the ''low Hsp70'' experimental group. Stimulation with LPS produced more variability in IL-10 levels between the various groups, although none of them reached statistical significance as compared to the ''vehicle'' group (**Fig. 4C, central panel**).


A
IFN-γ (pg/ml)
Untreated
+ αS
Vehicle
αS low
70 low
αS/70 low
αS
70 high
αS/70 high
IFN-γ (ng/ml)
+ LPS
αS high
Rel. IFN-γ (αS/LPS ratio)
B
IL-17 (pg/ml)
Rel. IL-17 (αS/LPS ratio)

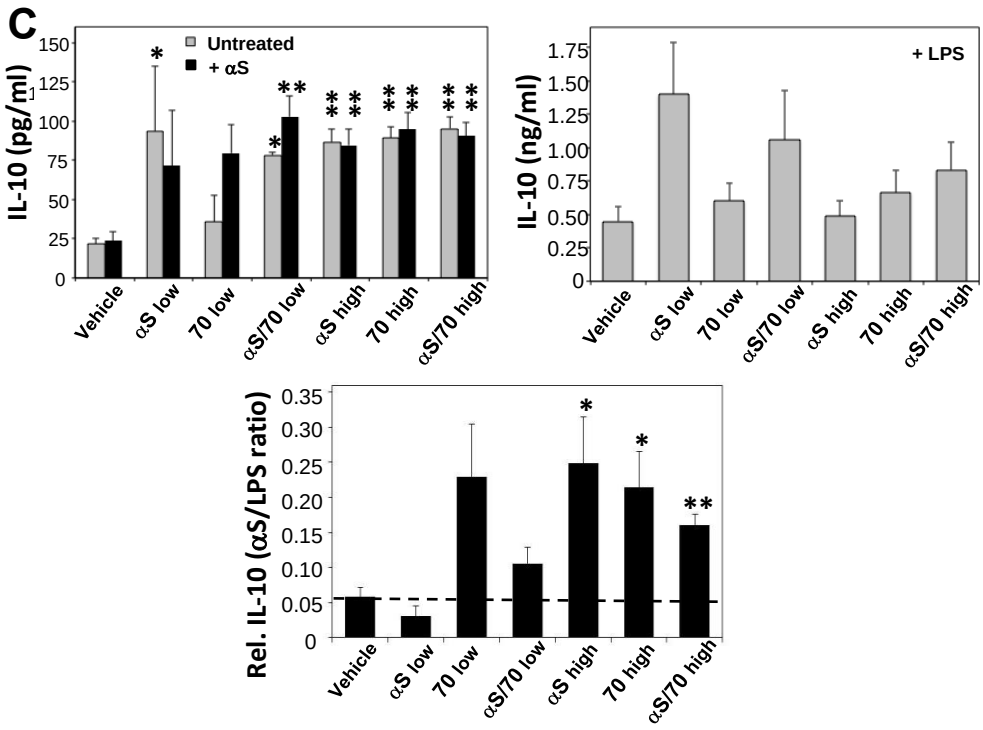



**Figure 4**. Cytokine release profile of splenocytes isolated from immunized mice. IFN-γ (A), IL-17 (B), andIL-10 (C) levels were measured in supernantants from cultured splenocytes isolated from immunized mice 24 hours after treatment with αSyn (20 µg/ml) or complete medium alone (left column), or with LPS (0,5 µg/ml) (center column). All cytokines were measured by ELISA. Pairwise αSyn/LPS ratios (right column) were calculated by dividing the cytokine levels elicited by αSyn (pg/ml) over the corresponding cytokine response to LPS (pg/ml) for each mouse. The detection limit for the IL-10/IFN-γ and IL-17 assay kits were <30 pg/ml, and 4 pg/ml, respectively. Represented values are mean ± S.E.M. (n = 5-7). Asterisks correspond to statistically significant differences between one particular group and the 'vehicle' group. Hash signs indicate statistically significant differences between different groups. */# P<0.05, **/## P<0.01.

In order to normalize the aSyn-specific response to the general splenocyte reactivity, we also analyzed the aSyn/LPS ratio for the three cyokines measured (**Fig. 4A, right panel**). A clear downregulation in the relative IFN-γ secretion levels upon challenge with aSyn could be observed for mice immunized with the aSyn/Hsp70 combination at both doses (three- to fivefold lower) and essentially unaltered relative IL-17 levels (**Fig. 4A, B, right panel**). Conversely, significant increases in the relative IL-10 levels upon aSyn challenge were seen for the ‘‘high aSyn’’ and ‘‘high Hsp70’’ experimental groups (five-fold higher), in addition to ‘‘high aSyn/Hsp70’’ combination (threefold higher) (**Fig. 4C, right panel**). Taken together, our results suggest that highly reactive splenocytes that are hypo-responsive toward aSyn and which display high IL-10 basal secreting levels, have been uniquely generated by immunization of mice with the aSyn/Hsp70 combination.

**IFNγ and IL-10 cytokine levels in serum indicate a shift toward an immunomodulatory response in aSyn/Hsp70-immunized mice**

To evaluate the biological relevance in vivo of immunization with the aSyn/Hsp70 complex, we assayed the levels of IFN-γ and IL-10 in serum obtained from immunized mice, one week after the booster (**Fig. 5**). While virtually all mouse groups showed no significant alterations in serum IFN-γ levels as compared to the vehicle group (12±5 pg/mL), the only significant change in this cytokine levels corresponded to a threefold reduction for the ‘‘high aSyn/Hsp70’’ combination (3.6±1.4 pg/mL) (**Fig. 5A**). Furthermore, the only significant change in serum IL-10 levels observed corresponded to a fourfold increase for the mouse group immunized with ‘‘high aSyn/Hsp70’’ (28±8 pg/mL) as compared to mice immunized with ‘‘vehicle’’ (7.0±1.8 pg/mL) (**Fig. 5A**). Finally, we analysed the pairwise serum IL-10/IFN-γ ratio (i.e., the IL-

10/IFN-γ measured levels for each mouse) and compared the different experimental groups. Remarkably, while the IL-10/IFN-γ ratio for the ''aSyn'' and ''Hsp70'' groups remains close to that observed for the ''vehicle'' group, immunization of mice with the ''aSyn/Hsp70'' combination at both doses produces a ca. eightfold increase in this ratio (**Fig. 5B**). This unique effect of the ''aSyn/Hsp70'' combination is evidenced by the shift in the serum IL-10/IFN-γ relative levels, from a pro-inflammatory, toward an immunomodulatory, profile (**Fig. 5B**).

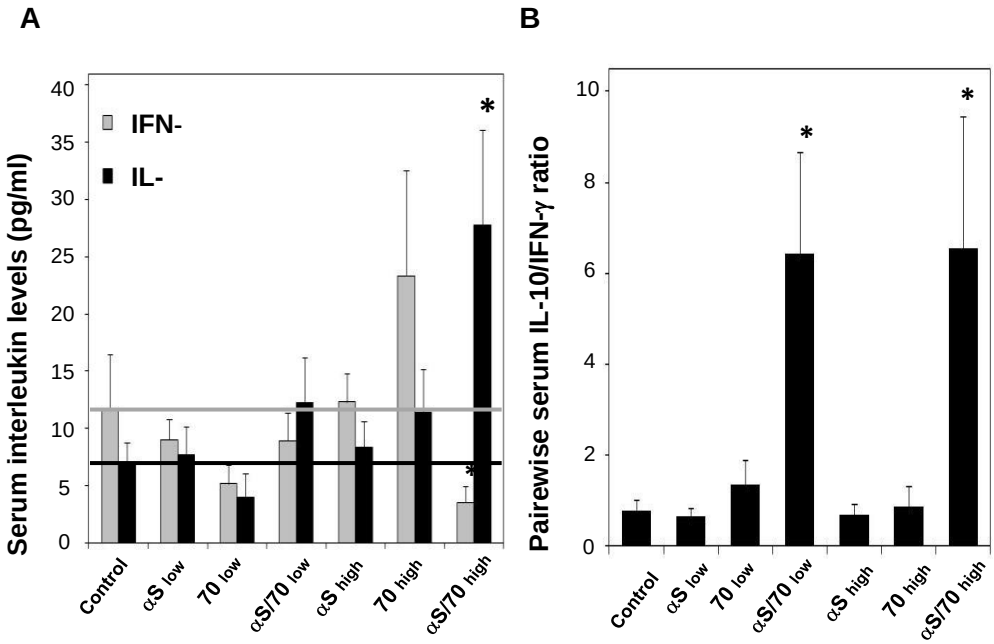



**Figure 5**. IFN-γ and IL-10 serum levels in immunized mice. IFN-γ (gray) and IL-10 (black) levels were measured in mouse sera one week after the end of the immunization protocol, by ELISA (A). Pairwise IL-10/IFN-γ ratios were calculated by dividing the measured IL-10 cytokine level by the measured IFN-γ cytokine level for each mouse (B). The detection limit for the IL-10 and IFN-γ assay kits were <30 pg/mL, Values are mean±S.E.M. (n=5–7). Asterisks correspond to statistically significant differences between one particular group and the "vehicle" group. * P<0.05, ** P<0.01.

## 5. Discussion

Over the last few years, immunotherapy has become an expanding subject of study as a novel approach for the treatment of neurodegenerative amyloid or misfolding diseases, which remain essentially incurable. Accumulated evidence highlights the central role in neurodegenerative disorders of an uncontrolled cellular-mediated response that could promote microglial activation and neuroinflammation, and ultimately lead to neurodegeneration (Mosley et al. 2012; Appel et al. 2010). In the present work we have combined the aggregating, IDP protein aSyn and the full-length and functional Hsp70 chaperone to promote the formation of an aSyn/Hsp70 complex in vitro, and used it to vaccinate naive mice in the absence of added adjuvants, to evaluate the resulting immune response.

Our findings reveal that, while increased levels of anti-aSyn IgM and IgG antibodies were produced by immunization with aSyn and Hsp70 proteins alone, no such effect was observed for the group vaccinated with the aSyn/Hsp70 combination, as compared to the control group. This unique effect of aSyn/Hsp70 immunization to produce a restrained anti-aSyn Ab response might be beneficial in the context of PD as it has been suggested that anti-aSyn antibodies are involved in the pathogenesis of the inherited form of the disease (Papachroni et al. 2007; Benkler et al. 2012; Chen et al. 1998). Moreover, this feature could be especially positive in the case of well-established autoimmune-related neurodegenerative diseases such as Multiple Sclerosis (MS) or Acute Motor Axonal Neuropathy, where elevated levels of specific IgM and IgG antibodies directed against certain self-antigens have been shown to contribute to disease onset and progression (Zhang et al. 2005; Huizinga et al. 2007; Beltrán et al. 2012; Sádaba et al. 2012; Shahrizaila et al. 2014).

A similar trend was seen for the Treg and Teff cell populations, in which the increments and reductions, respectively, elicited by immunization with aSyn and Hsp70 alone were restrained as a result of vaccination with the aSyn/Hsp70 complex, again indicating a differential effect of the combined proteins and the formation of an immune-functional complex. This ‘‘buffering’’ of the regulatory and non-regulatory/effector T cell content toward basal levels as a result of immunization with the aSyn/Hsp70 complex – as opposed to immunization with both proteins separately – could be potentially beneficial as it indicates a capacity of the aSyn/Hsp70 immunization to restore or maintain the Treg/Teff equilibrium in a disease, or preonset, scenario.

Our results with cultured splenocytes from immunized mice and subsequent challenge with aSyn or LPS, show clearly that the aSyn-specific response is suppressed in the case of vaccination with the aSyn/Hsp70 complex when compared to vehicle-injected mice, as indicated by lower aSyn-specific IFN-γ and IL-17 relative secretion levels. Even though other cells types present within splenocytes could also respond to LPS stimulation, in addition to T cells – such as B cells or dendritic cells – this result may reflect a lower Th1/Th17 response (a T cell-mediated response involving the liberation of IFN-γ and IL-17) toward aSyn as a result of immunization with the aSyn/Hsp70 complex. In addition, we found that this change is accompanied by higher basal levels of secreted IL-10 that are not significantly altered when challenged with aSyn.

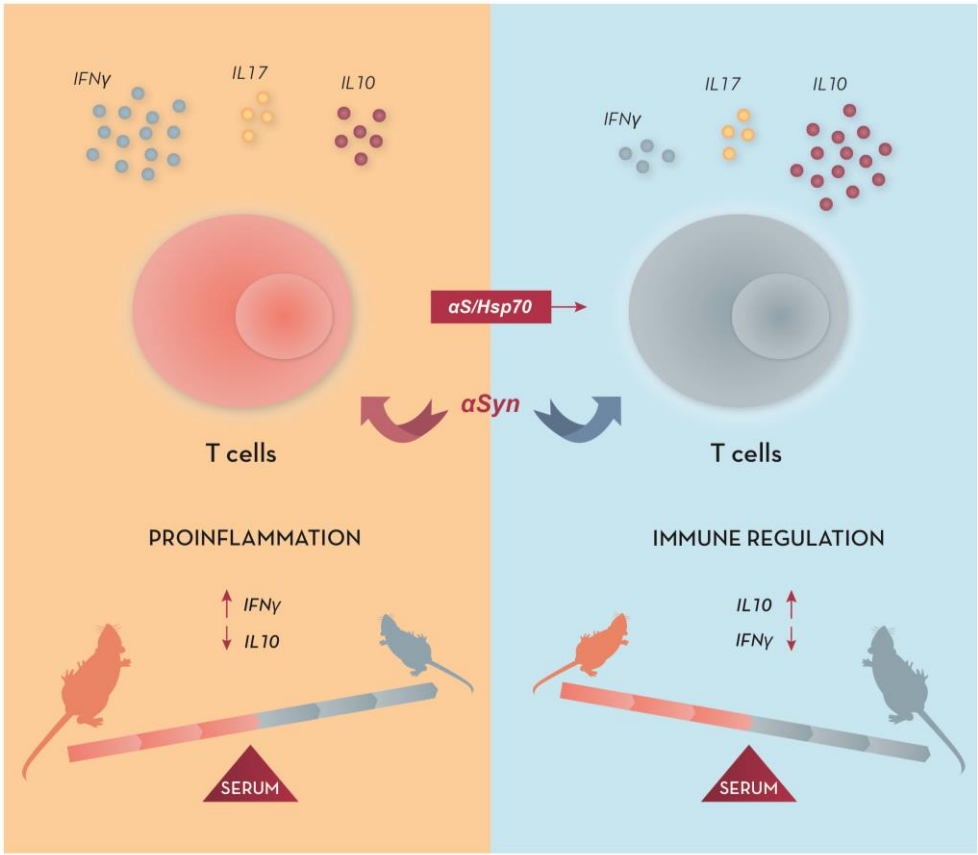
Finally, we found that immunization with the aSyn/Hsp70 complex uniquely produced changes in the cytokine levels in serum, consisting of a threefold reduction of IFN-γ and a fourfold increase of IL-10 absolute levels, as well as a six-fold increase in the pairwise IL-10/IFN-γ ratio, as compared to vehicle-injected mice. These results clearly demonstrate that the aSyn/Hsp70 combination produces a shift in the peripheral immunity toward a modulatory

– and presumably protective – phenotype. This effect could potentially be highly beneficial for treatment against misfolding diseases and other neurodegenerative disorders, as the Th1/Th17 response has been linked to microglia polarization and/or maintenance toward an M1 (classical) pro-inflammatory phenotype (Mount et al. 2007; Kebir et al. 2007), which in turn has been shown to enhance neurodegeneration in PD (Reynolds et al. 2010; Wu et al. 2002; Batchelor et al. 1999).

Overall, our results show that human Hsp70 chaperone acts as a cell immunity adjuvant in combination with full-length aSyn, producing a shift in the immune response characterized by a restrained aSyn-specific humoral immunity coupled to a modulatory/protective phenotype, in immunized naive mice (**Scheme 1**). This shift toward an immunomodulatory phenotype cannot, in principle, be attributed to an increase in the Treg cell content, as similar levels of Treg cells from spleen were measured in the aSyn/Hsp70-immunized mice as compared to the control group. Therefore, either a different quality of Treg cells, or the engagement of other cell types such as NKT cells, B lymphocytes or even dendritic cells, could potentially be involved in this particular phenotype.

With the aim of minimizing the potential adverse effects of immunization, such as exacerbated inflammation or autoimmunity, two aSyn-based vaccination approaches designed to bypass the antigen-specific cellular immunity completely, while promoting a strong antigen-specific humoral response, have been recently reported (Ghochikyan et al. 2014; Mandler et al. 2014). In this work we describe an alternative strategy that is based on the use of a combination of Hsp70 and aSyn to produce a restrained anti-aSyn humoral response coupled to an immunomodulatory phenotype. We propose that such a combined response might simultaneously tackle two aspects of PD pathobiology at the onset and progression stages: firstly, an immune-regulatory

phenotype in the periphery is expected to exert a neuroprotective effect by communicating and interacting with chronically activated microglia and other immunocompetent cells in the CNS, and therefore counteracting neuro-inflammation. Secondly, it might prevent the development of potentially detrimental polyclonal antibody responses against aSyn in an aSyn overexpression or aggregation scenario, as opposed to a specific antibody response directed toward toxic oligomeric species or epitopes, of aSyn.




**Scheme 1**. Immunization with aSyn/Hsp70 produces a shift from a proinflammatory profile toward an immunomodulatory profile. Immunization of mice with the aSyn/Hsp70 complex in the absence of added adjuvant generates a unique response

consisting of highly reactive splenocytes toward a nonspecific/ polyclonal insult which display higher IL-10 basal secreting levels, and which are hypo-responsive toward aSyn. In vivo, immunization with aSyn/Hsp70 produces a restrained anti-aSyn Ab humoral response, as compared to immunization with aSyn alone. This effect is accompanied by unique changes in IL-10 and IFN-γ cytokines serum levels, consisting of higher immunomodulatory IL-10 and lower Th1-linked IFN-γ, and producing clear-cut shifts in the serum IL-10/IFN-γ relative levels. Overall, immunization with the "aSyn/Hsp70" combination generates a shift in cellular immunity from a proinflammatory profile, toward an immunomodulatory phenotype.

Furthermore, we suggest that the exploitation of the differential immune response elicited by specific combinations of aggregating proteins with immune-active and functional chaperones, or by certain HSP/aggregating protein complexes, could be an effective means of suppression of the uncontrolled pro-inflammatory environment associated to neurodegeneration in amyloid and other misfolding disorders.

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## 7. Author Contributions

ALG, DP, and CR designed the experiments and interpreted the data. ALG, MCG, RK, and MML performed the experiments with contributions from EJDG, JV, and JJTA. EJDG provided the a-synuclein protein. LTG designed and performed the Biacore experiments, and analysed the Biacore data. ALG, DP, and CR wrote the manuscript with contributions from CMD, EJDG, and JJTA. CR supervised all the experiments.

# Chaperome screening leads to identification of Grp94/Gp96 and FKBP4/52 as modulators of the α-synuclein–elicited immune response

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## 1. Abstract

We have investigated the potential role of molecular chaperones as modulators of the immune response by using α-synuclein (aSyn) as an aggregation prone model protein. We first performed an in vitro immunoscreening with 21

preselected candidate chaperones and selected 2 from this set as displaying immunological activity with differential profiles, Grp94/Gp96 and FKBP4/52. We then immunized mice with both chaperone/α-synuclein combinations using monomeric or oligomeric α-synuclein (MaSyn or OaSyn, respectively), and we characterized the immune response generated in each case. We found that Grp94 promoted aSyn-specific T-helper (Th)1/Th17 and IgG1 antibody responses (up to a 3-fold increase) with MaSyn and OaSyn, respectively, coupled to a Th2-type general phenotype (generating 2.5- fold higher IgG1/IgG2 levels). In addition, we observed that FKBP4 favoured a Th1-skewed phenotype with MaSyn but strongly supported a Th2-type phenotype with OaSyn (with a 3-fold higher IL-10/IFN-g serum levels). Importantly, results from adoptive transfer of splenocytes from immunized animals in a Parkinson's disease mouse model indicates that these effects are robust, stable in time, and physiologically relevant. Taken together, Grp94 and FKBP4 are able to generate differential immune responses to α-synuclein–based immunizations, depending both on the nature of the chaperone and on the aggregation state of α-synuclein. Our work reveals that several chaperones are potential modulators of the immune response and suggests that different chaperones could be exploited to redirect the amyloid-elicited immunity both for basic studies of the immunological processes associated with neurodegeneration and for immunotherapy of pathologies associated with protein misfolding and aggregation.

**Key Words**: misfolding/amyloid disease • Parkinson • heatshock protein • immunotherapy

**Abbreviations**: aSyn, α-synuclein; AT, adoptive transfer; AU, arbitrary units; BSA, bovine serum albumin; EU, endotoxin unit; HSP, heat-shock protein; MaSyn, monomeric α-synuclein; MPTP, 1-methyl-4-phenyl-1,2,3,6-

tetrahydropyridine; OaSyn, oligomeric α-synuclein; PD, Parkinson's disease; Teff, effector T; Th, T-helper; Treg, regulatory T

## 2. Introduction

The eukaryotic chaperome has been defined as an interconnected network composed of a large number of molecular chaperones and co-chaperones, which are essential for de novo protein folding and to prevent protein misfolding and aggregation under heat-shock or environmental stress (Albanèse et al. 2006). In addition to the long-established chaperoning functions of heat-shock proteins (HSPs) with their client polypeptides (Hartl et al. 2011), certain HSPs have lately been reported to play diverse roles as modulators of innate and adaptive immunity (Binder 2014). Such emerging activities, which include promoting dendritic cell maturation, exerting immunosuppressive signals, facilitating the activation of lymphocytes and macrophages, and promoting antigen cross-presentation and T-cell priming (Binder 2014; Pockley et al. 2008; Murshid et al. 2012; Srivastava 2002), have mainly been studied with tumor antigens and peptides, and exogenously administered HSPs are currently being tested for the treatment of cancer, autoimmune disease, and a variety of infections (Srivastava 2012). Recently, as a result of exploiting both features, its immunogenic and chaperoning activities, we have reported that Hsp70 is able to redirect the immune response elicited by α-synuclein (aSyn), a protein whose misfolding and aggregation are linked to neurodegenerative diseases, toward a T-helper (Th)2-type immunity (Labrador-Garrido et al. 2014).

Misfolding or amyloid disorders, such as Parkinson's disease (PD), Alzheimer's, and Huntington's diseases, and also other conditions including type II diabetes and spongiform encephalopathies, are characterized by the conversion of an

initially soluble and functional polypeptide into oligomeric intermediates and then into insoluble fibrillar aggregates (Chiti & Dobson 2006). In each particular disorder, unfolding and/or misfolding of specific aggregation-prone proteins are thought to be key initial steps in the development of disease (Knowles et al. 2014). In the case of PD and other synucleinopathies, the aberrant aggregation of aSyn in certain regions of the brain ultimately produces insoluble amyloid-like fibrils linked to pathogenesis and disease progression (Marques & Outeiro 2012).

A common pathologic feature of neurodegenerative misfolding disorders is sustained microglial activation and neuroinflammation in the central nervous system, both of which are currently thought to involve mechanisms mediated by peripheral immunity (Appel et al. 2010; Glass et al. 2010). Nevertheless, the question regarding which immunological profiles are protective and which lead to neurodegeneration along the course of disease has not been completely resolved (Appel et al. 2010; Glass et al. 2010). Importantly, a link has been established between aSyn and the abnormal immunologic process that accompanies the onset and progression of synucleinopathies, which includes alterations in the T-cell–mediated immunity in the brain and in the peripheral system (Allen Reish & Standaert 2015; Sanchez-Guajardo, Barnum, et al. 2013; Roodveldt et al. 2011). Not surprisingly, several attempts have been made to induce a protective immune response against PD and other misfolding/amyloid disorders, including immunization with aSyn in mouse models of disease (Masliah et al. 2005; Masliah et al. 2011; Valera & Masliah 2013; Sanchez-Guajardo, Annibali, et al. 2013; Ghochikyan et al. 2014; Benner et al. 2008; Mandler et al. 2014).

Thus far, the potential of the chaperome as a modulator of the immune response has not been reported. The aims of the present study were, first, to

interrogate the chaperome on its immunomodulatory capabilities—and their degree of diversity—in relation to aSyn, a typical misfolding/amyloidogenic protein, and second, to characterize in detail the immunological profiles promoted by chaperoned aSyn as a result of immunization in mouse. Such an insight could be highly relevant for future studies on the physiopathology and immunotherapeutic strategies on PD and other related disorders. To those aims, we first identified those members of the chaperome, which, according to previous reports, had either been linked to aSyn or PD, or alternatively had been found to display immunological activity.

We then applied an in vitro screening strategy using a set of 21 preselected candidates to identify immune-functional aSyn/chaperone combinations. Two chaperones showing different profiles in vitro, Grp94/Gp96 and FKBP4/52, were selected and used in aSyn-based immunization protocols using either monomeric (MaSyn) or oligomeric (OaSyn) forms of aSyn in C57BL/6mice, and the resulting immune responses were characterized by assaying the different T-cell populations, humoral antibody responses, and serum cytokine profiles.

Remarkably, the results revealed very significant differences in the immunological phenotype in each case, depending both on the chaperone employed and on the aggregation state of aSyn. This study, therefore, indicates that a variety of molecular chaperones, including Grp94/Gp96 and FKBP4/52, can modulate the immunity generated by a misfolding or amyloidogenic protein such as aSyn, and they do so by supporting different immunologic phenotypes.

## 3. Material and Methods

### Source of molecular chaperones and preincubation buffers used for in vitro screening

All proteins were human, recombinant, and highly purified: Hsp70 (HSPA1A, #10-054-165238), 14-3-3γ (#10-002-38082), 14- 3-3ε (#10-002-38083), and 14-3-3τ (#10-002-38084) were obtained from GenWay Biotech (San Diego, CA, USA); Hsc70 (HSPA8, #ab78431), Grp75/mortalin (HSPA9, #ab79145), Hsp90β (Hsp90B, #ab80353), STUB1/CHIP (HSPABP2, #ab82791), FKBP12 (#ab85840), cyclophilin A (CypA, #ab96022), DJ-1 (PARK7, #ab51198), apolipoprotein-J/clusterin (APO-J, #ab69754), Pin1 (#ab51230), Hsp105 (HSPH1, #ab78790), and cyclophilin 40 (Cyp40, #ab78815) were obtained from Abcam (Cambridge, United Kingdom); FKBP4/52 (#NBC1-22936), Hsp27 (HSPB1, #NBP1-30308), Rab11A (#NBP1-44381), Hsp40 (DNAJB1, #NBC1-18369), and Grp94/Gp96 (HSP90B1, #NBC1-21058) were obtained from Novus (Cambridge, United Kingdom). Highly purified Hip (ST13) protein was a kind gift of Prof. Jose M. Valpuesta's laboratory (Centro Nacional de Biotecnologia, Madrid, Spain). All purchased recombinant proteins contained an endotoxin level of <1 endotoxin unit (EU)/μg protein.

Buffer conditions used for preincubating the chaperone/aSyn combinations, or the corresponding proteins alone as references, were as follows: buffer 1, 25mMTris, 150mM KCl, 5mM $MgCl_2$, pH 7.4 (Hsp70 and Hsc70); buffer 2, 20 mM Tris, 100 mM NaCl, pH 8.0 (Grp75, Hsp90β, CHIP/STUB1, FKBP12, CypA, DJ-1, Pin1, and Hsp105); buffer 3, 20mM HEPES, 150mM KCl, 1mM DTT, 1 mM PMSF, 5% glycerol, pH 7.5 (Hip); buffer 4, 20 mM Tris, pH 7.4 (14-3-3γ, 14-3-3ε, 14-3-3τ, and Cyp40); buffer 5, 20 mM Tris, pH 8.0 (FKBP4/52, clusterin, Hsp27, Rab11A, Hsp40, and Grp94). In the cases of Hsp70, Hsc70, and Hsp90β, 4 mM

ATP and, 2 h later, 2.5 mM ADP, were added to the preincubation mixtures and the corresponding controls.

**Overexpression and purification of aSyn and preparation of aSyn oligomers**

Human wild-type aSyn was overexpressed in *Escherichia coli* BL21(DE3) cells using plasmid pT7-7 and purified as described previously (Roodveldt et al. 2010). Before lyophilization, the aSyn solution was passed through a 100 kDa cutoff Amicon Ultra membrane, 0.5 ml (Millipore, Billerica, MA, USA) to remove any aSyn aggregates or bound endotoxins. The purity and monomeric state of the aSyn preparation (>95%) was assessed by 15%SDS-PAGE, mass spectrometric analysis, and 4–10% native PAGE (Lonza, Basel, Switzerland), as previously described (Roodveldt et al. 2010). To remove endotoxin, the aSyn solution was passed through a 100 kDa cutoff filter, before being lyophilized and stored at -80°C. The preparation and characterization of soluble aSyn oligomers were carried out as reported previously (Roodveldt et al. 2012; Roodveldt et al. 2013) with human A53T aSyn and the purified oligomeric fractions in PBS were stored at 4°C and used within 4 h. Endotoxin levels in the protein preparations were measured by the ToxiSensor Chromogenic LAL Assay kit (GenScript, Piscataway, NJ, USA), and the values obtained were <1 EU/mg protein. The concentrations of non-aggregated and OaSyn were determined by means of Micro BCA Reagent kit (Pierce, Rockford, IL, USA).

**Isolation of murine splenocytes**

Splenocytes were isolated from excised mice spleen by perfusing with 10 ml of PBS, after which the erythrocytes were lysed by osmotic shock. In the case of splenocytes used for *in vitro* screening, cells from 4 non-immunized mice were

pooled together. In all cases, the number of cells was determined by counting in a hemocytometer.

### *In vitro* immunoscreening of chaperones and aSyn/chaperone combinations

Splenocytes were isolated from the spleen of 4 nonimmunized 5- to 7-wk-old C57BL/6 male mice and pooled together, as previously described (Labrador-Garrido et al. 2014). Cells (3x $10^6$ per well) were cultured in a 12-well plate in RPMI medium (BioWhittaker, Verviers, Belgium) with 10% inactivated fetal bovine serum (BioWhittaker). Before cell culture treatment, all proteins alone or in combination, were preincubated for 4 h at room temperature at 10 times their final concentrations in their corresponding buffer as detailed above. After diluting each mixture 10 times in culture medium containing 10 μg/ml polymixin B (Sigma-Aldrich, St. Louis,MO,USA), each well with cultured splenocytes was treated either with medium alone (control), with chaperone at a concentration of 140nM(except for FKBP12, cyclophilinA,DJ-1, and Pin1, which were used at 715 nM because of their lower immunogenicity, and Hsp105 and Grp94/Gp96, which were used at 14 nM because of their higher immunogenicity), with a combination of each chaperone and aSyn at equimolar concentrations, or with aSyn alone (at 140, 715, or 14 nM, accordingly). After incubation for 24 h, the cell culture supernatants were collected and centrifuged at 500 g for 5 min to separate the cells, and $10^6$ cells were labeled with anti–CD86-FITC and anti–I-A/I-E-PE antibodies (BD Biosciences, San Diego, CA, USA) by following the manufacturer's instructions. They were then analysed by flow cytometry using a FACS Calibur cytometer with CellQuest Pro (BD Biosciences) software. Two independent experiments were performed, both containing sample duplicates. The cell-free supernatant solutions were stored at -80°C for subsequent cytokine assaying.

**Overexpression and purification of FKBP52 and Grp94 proteins used for immunization procedures**

Highly purified, recombinant Grp94/Gp96 protein (canine, #ab92290) overexpressed with baculovirus was purchased from Abcam. Highly purified, recombinantFKBP4/52 (FKBP59,human) was produced and purified as previously described (27). Briefly, the latter protein was overexpressed in *E. coli* as a gluthathione- S-transferase fusion and purified on Glutathione Sepharose (GE Healthcare Life Sciences, LittleChalfont,United Kingdom) equilibrated in PBS. Unbound material was washed from the column with 10mM ATP-magnesium salt (Sigma-Aldrich) in PBS and then with PBS containing 1 mM DTT. FKBP52/4 was eluted from the column following cleavage from gluthathione-S-transferase with bovine thrombin. The protein was subsequently purified by gel filtration on a G200 Sepharose HR26/60 column equilibrated in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl and then on a Mono Q HR10/10 column (GE Healthcare Life Sciences) binding in 20 mM bis-Tris (pH 6.2) containing 1 mM DTT and eluting with a linear gradient from 0 to 1 M NaCl over 180 ml. Fractions containing the protein were analysed by SDS-PAGE and dialyzed overnight in 50 mM TrisHCl (pH 7.4) containing 1mM DTT. Purified FKBP52 was incubated with polymixin B-agarose (Sigma-Aldrich) preequilibrated with buffer containing 1 mM DTT, according to the manufacturer's instructions, to remove endotoxins. Endotoxin levels in the purified protein preparations were measured by the ToxiSensor Chromogenic LAL Assay kit, and the values obtained were <1 EU/mg protein.

**Animals**

Male C57BL/6 mice 6–7 wk old were purchased from the University of Seville Center for Animal Production and Experimentation (Espartinas, Seville, Spain).

Animals were kept for 1 wk in the local animal house before the start of the immunization protocol, to allow the mice to acclimatize to their new environment. At all stages of the study, animals from each experimental group were allocated into different cages such that each cage contained up to 5 mice, in every case corresponding to a mix from different experimental groups. All animal procedures were in accordance with good animal practice as defined by the relevant national/EU and Animal Research: Reporting of *In Vivo* Experiments guidelines and the ´Ético de Experimentación Animal–Centro Andaluz de Biología Molecular y Medicina Regenerativa -Andalusian Molecular Biology and Regenerative Medicine Centre (CEEA–CABIMER) Experimental Animal Committee, and were approved by the corresponding committee (CEEA-2010-14).

**Protein uptake and splenocytes cytokine secretion assays**

Purified A90C aSyn variant and bovine serum albumin (BSA; Sigma) labelled with Alexa Fluor 647 (AF647) in PBS were used. For protein labelling, purified aSyn was first passed over a PD10 desalting column equilibrated in PBS (GE Healthcare Life Sciences, Buckinghamshire, United Kingdom) to remove 10 mM DTT present in the stock protein solution to keep the cysteine residue reduced. The BSA was dissolved in PBS at 2 mg/ml and both proteins were labelled with AF647 *via* amine coupling. Reactions were carried out at a 1.5 molar excess of label to protein. The excess label was separated from the protein *via* gel filtration using a PD10 column.

In the protein uptake assay, murine splenocytes in culture were treated either with medium alone (control) or with preincubation mixtures containing the selected chaperones (Hsp70, Grp94/Gp96, or FKBP4/52) at a final concentration of 420 nM, or a combination of each chaperone and labelled

client protein (aSyn or BSA) at equimolar concentrations, or labelled client protein (aSyn or BSA) alone, at the same concentration as described above. Before being added to the cell culture, preincubation mixtures were prepared as described in the screening procedures. After incubation for 1 h, cells were harvested and processed for flow cytometry analysis for MHC-II and labeled client protein detection.

**Immunization protocols**

**Immunizations with MaSyn (protocols 1 and 2) and with BSA**

Preincubation mixtures were prepared forGrp94 and/or MaSyn in 20 mM HEPES (pH 7.5) for 2 h at 42°C (28), and for FKBP52 and/or MaSyn in 20 mM Tris (pH 8.0) for 2 h at 30°C, keeping a 1:1 molar ratio for chaperone:MaSyn. Afterward, the preincubated mixtures were diluted in PBS (approximately 50-fold dilution of the preincubation mixtures) in the absence of added adjuvant. Mice were immunized with the different preparations, as follows: Grp94 (5 μg) and/or MaSyn (0.8 μg) (protocol 1), FKBP52 (30 μg) and/orMaSyn (8.6 μg) (protocol 2). Mice were injected on d 0 with a single 100 μl s.c. injection in the lumbar region. The same procedure was repeated on d 7. On d 14,mice were killed, and the spleen and 500–700 μl of blood were extracted for analyses. In addition, removal of endotoxin from the Hsp70 preparation and immunizations with Hsp70/aSyn, Hsp70, aSyn, vehicle/buffer (n = 5), BSA and Hsp70/BSA(n=3) were done as previously described (8). BSA (Fraction V; Sigma- Aldrich) solution was previously depleted of endotoxin by passing the solution through a 100 kDa cutoff filter by centrifugation. Endotoxin levels in the protein preparations were measured by the ToxiSensor Chromogenic LAL Assay kit (GenScript), and the values obtained were <1 EU/mg protein.

**Immunizations with OaSyn (protocol 3)**

Preincubation mixtures were prepared as described above for protocols 1 and 2. Mice were immunized with Grp94 (8 μg) and/or OaSyn (13 μg) or FKBP52 (9.4 μg) and/or OaSyn (13 μg) to generate molar ratios of 1:10 and 1:5 chaperone:aSyn, respectively. All preparations were diluted in PBS (~50-fold dilution of the preincubation mixtures) in the absence of added adjuvant. On d 0, mice were injected with a single 100 μl s.c. injection in the lumbar region. The same procedure was repeated on d 7 and 21. On d 28, the mice were killed, and the spleen and 500–700 μl of blood was extracted for analyses.

**Determination of $CD4^+$, regulatory T-cell, and effector Tcell populations in isolated splenocytes**

After isolation of splenocytes as described above, $10^6$ cells were labeled with anti–CD4-FITC, anti–CD25-APC, and anti–Foxp3-PE antibodies (BD Biosciences), by following the manufacturer's instructions. Flow cytometry analysis was performed with a FACS Calibur cytometer using CellQuest Pro(BDBiosciences) software. The $CD4^+$ cell populationwas calculated as the percentage of cells positive for CD4 within the total lymphocyte population. The regulator T ($T_{reg}$) cell population was calculated as the percentage of cells positive for CD4, CD25, and Foxp3 staining ($CD4^+CD25^+Foxp3^+$) among the $CD4^+$ lymphocyte population. The effectorT ($T_{eff}$) cell (nonregulatory) population was calculated as the percentage of cells that stained positively for CD4 and negatively for Foxp3 ($CD4^+Foxp3^-$) among$CD4^+$ lymphocytes.

**Adoptive transfer of murine splenocytes and 1-methyl-4- phenyl-1,2,3,6-tetrahydropyridine treatment of acceptor mice**

After death, a pool of splenocytes from 5 immunized mice was prepared by mixing 2 x $10^7$ cells per mouse for every experimental group. On d 0, each mouse was injected intraperitoneally with $10^7$ cells. A total of 5 acceptor mice per group were used (n= 5). On d 7, acceptor mice were placed on a 1-methyl-4-phenyl- 1,2,3,6-tetrahydropyridine (MPTP) treatment to model chronic PD, as previously described (Muñoz-Manchado et al. 2013). One month after the end of the MPTP treatment, the mice were killed and 500–700 µl of blood was extracted for analyses.

***In vitro* stimulation of splenocytes**

Splenocytes were isolated from the spleen of immunized mice after killing, as previously described. Cells (2 x $10^6$) from each mouse were divided and cultured in 2 wells (of a 12-well plate) in RPMI medium (BioWhittaker) with 10% inactivated foetal bovine serum. Each well was treated either with medium alone (control) or with aSyn (20 µg/ml). After incubation for 24 h, the supernatant solutions were collected and centrifuged at 500 g for 5 min to eliminate any remaining cells and debris, and stored at -80°C for subsequent cytokine assaying.

**Antibody determination by ELISA**

Blood samples extracted after death were left for 1 h at 4°C and 1 h at room temperature to allow clotting to occur. After clot formation, the samples were centrifuged at 21,000 g for 15 min to obtain cell-free serum, and stored at -80°C for further analyses. Levels of anti-aSyn IgM or IgG antibodies and total IgM or

IgG antibodies were quantified as described previously (Labrador-Garrido et al. 2014). The values shown [expressed in arbitrary units (AU)] were calculated as follows: the values of anti-aSyn result from multiplying the absorbance measured in the ELISA assay by the dilution factor of the samples; the values of total antibodies result from multiplying the measured absorbance by the dilution factor of the samples and by a factor of 1023; finally, the anti-aSyn/total antibody ratios (AU) were calculated by dividing the absorbance values obtained in the corresponding ELISA assays (anti-aSyn antibodies over total IgG antibodies, previously multiplied by each dilution factor), and finally multiplying by a factor of 105 for simplicity purposes.

IgG1 and IgG2a isotypes were quantified by following the same procedure as for total IgG antibodies, except that Rat monoclonal anti-mouse IgG1 (Abcam) and Rat monoclonal antimouse IgG2a (Abcam), respectively, were used as detection antibodies.

**Cytokine determination by ELISA**

For quantification of IFN-γ, IL-10, and IL-17 levels from culture supernatants or serum, specific ELISA kits, namely mouse IFN-γ and mouse IL-10 BD OptEIATM kits (BD Biosciences), and ELISA Development Kit Murine IL17 (PreproTech, London, United Kingdom), were used according to the manufacturer's instructions.

**Statistical analyses**

Statistical analysis was performed by using the IBMSPSS Statistics 20 pack (Chicago, IL, USA). For all parameters (T-cell populations, antibody determinations, and cytokine measurements), the Kruskal-Wallis 1-way ANOVA

was first performed to evaluate the existence of significant differences among the experimental groups. To determine the differences between groups and to obtain the P values, the nonparametric Mann-Whitney U test for 2 independent samples was performed. Each group consisted of 5 mice. Statistically significant differences were those with $P<0.05$.

## 4. Results

### In vitro screening of a set of chaperone candidates for the selection of immunofunctional aSyn/chaperone combinations

As a first step in identifying promising candidates that could potentially serve to immunochaperone aSyn, we first designed and set up an in vitro assay based on cultured murine splenocytes to screen a large set of preselected candidates. The preselected set consisted of 21 chaperones or co-chaperones (**Table 1**), all of which had either been linked to aSyn or to PD or had been found to display immunological activity, according to previous reports.

Prior to the screening, mixtures of highly purified aSyn and chaperone proteins (or the corresponding controls with each protein alone) were prepared as described in Materials and Methods. Following a 4 h incubation at room temperature, murine splenocytes in culture were stimulated with the various aSyn/chaperone combinations and protein controls, previously diluted in PBS to their final concentrations (see Materials and Methods) and incubated at 37°C for 24 h. Stimulated splenocytes were subsequently labeled to enable detection of CD86 and MHC-II surface markers and analysed by flow cytometry (**Table 1**). We then plotted the percentages of CD86+/MHC-II+ cells obtained according to the different combination:chaperone ratios vs. the corresponding combination:aSyn ratios, showing the changes produced by the combination of

aSyn and each chaperone, and observed a dispersion of data for the various chaperone proteins that we tested (**Fig. 1**). In addition, the levels of 3 key cytokines, namely IFN-γ, IL-10, andIL-17, were assayed in the culture supernatants (**Table 1**) and plotted in an analogous manner (**Figs. 1B–D**). We designed our screening procedure to favour highly immunogenic chaperone/aSyn combinations with a diversity of modulatory profiles while relegating those with extreme IL-17 responses, which may promote cytotoxic Th17 cell-mediated effects (Reynolds et al. 2010; Zhang et al. 2013). Based on this, we identified chaperones that showed the highest signal ratios along one or both axes in the plots representing MHC-II+/CD86+ expression, and IL- 10 and IFN-γ cytokine levels, while a negative selection criterion was applied for IL-17 cytokine (**Fig. 1** and **Table 1**). Three chaperones, Grp94/Gp96 (HSPC4), Hsp105, and peptidyl-prolyl cis-trans isomerase FKBP4 (or FKBP52/59), were found to produce the largest changes of CD86+/MHCII+ cells after splenocyte stimulation (Fig. 1A). The highest signal ratios for IFN-γ were observed for Cyp40, Pin1, Grp94/Gp96, FKBP4/52, Rab11A, Hsp40, and Hsp105. On the other hand, the highest signal ratios for IL-10 were observed for Grp94/Gp96, FKBP4/52, clusterin, and Hsp105 (**Table 1**). And, finally, the highest signal ratios for IL-17 were observed for 14-3-3ε, 14-3-3τ, and Hsp90β. Next, the chaperones that exhibited the largest values in combination with aSyn for the first 3 parameters measured and that did not stand out in the IL-17 assay were identified (**Table 1**). Based on these analyses, Grp94/Gp96 and FKBP4/52 were selected as promising candidates to chaperone aSyn in our subsequent studies.

| Chaperones | | MHC-II$^+$/CD86$^+$ (%) | | | IFNγ (pg/ml) | | |
|---|---|---|---|---|---|---|---|
| *Protein name* | *Altern.name* | *αSyn* | *Chap.* | *Comb.* | *αSyn* | *Chap.* | *Comb.* |
| **HSPA1A** | **Hsp70** | 3.5±0.55 | 8.45±0.14 | 9.9±0.13 | 31.8±8.9 | 27.3±8.8 | 50.5±3.8 |
| **HSPA8** | **Hsc70** | | 11.8±2.37 | 15.5±2. | 20.1±3.8 | 708±42.1 | 565±68 |
| **HSPA9** | **Grp75** | | 14.2±0.07 | 12.6±1.4 | | 1879±87 | 1839±88 |
| **HSP90B** | **Hsp90β** | | 5.32±0.29 | 4.1±0.4 | | 20. 6±1.9 | 43.4±6.6 |
| **STUB1** | **CHIP** | | 14.2±0.13 | 13.5±0. | | 1691±201 | 1842±59 |
| **Cyp40** | | 1.6±0.1 | 3.5±0.27 | 5.7±0.11 | 37. 5±8.9 | 41±17.3 | 730±148 |
| **FKBP1A** | **FKBP12** | 2.8±0.5 | 4.8±0.30 | 5.9±0.6 | 18.4±1.5 | 18.1±2.1 | 57±14 |
| **CypA** | | | 3. 9±0.16 | 6.3±0.03 | | 23.5±12.3 | 16.6±4.0 |
| **PARK7** | **DJ- 1** | 2.2±0.12 | 4.7±0.18 | 5.2±0.2 | 43.4±8.7 | 1393±96 | 1363±103 |
| **PIN1** | **Pin1** | 2.8±0.5 | 3.32±0.03 | 4.8±0.4 | | 12. 8±1.4 | 189±29.6 |
| **HSPC4** | **Grp94/Gp96** | 2.4±0.3 | 7.6±0.8 | 15.1±0.5 | 20.3±3.75 | 490±55 | 1093±12 |
| **14-3-3γ** | | 2.4±0.55 | 1.68±0.06 | 2.5±0.3 | | 14.3±0. 6 | 23.2±8.8 |
| **14-3-3ε** | | 1.4±0.2 | 1.35±0.06 | 1.7±0.07 | 34.2±3.8 | 11.9±0.1 | 31.7±4.2 |
| **14-3-3t** | | | 1.54±0.05 | 1.9±0.5 | | 15.0±0.4 | 24.8±2.7 |
| **ST13** | **Hip** | 2.2±0.1 | 5.8±0.22 | 5.8±0.1 | 47.3±7.7 | 40.7±4.9 | 43. 6±4.2 |
| **Apo-J** | **Clusterin** | | 1.5±0.16 | 2.2±0.3 | | 23.7±2. 7 | 60.0±4.4 |
| **HSPB1** | **Hsp27** | 0.8±0.06 | 0.78±0.09 | 1.6±0.1 | 16.7±2.3 | 13.6±0.2 | 40.0±5.7 |
| **FKBP4** | **FKBP52/59** | | 12.9±0.1 | 12.3±0.1 | | 6080±318 | 6430±124 |
| **RAB11A** | | 2.3±0.2 | 12.7±4.5 | 7.7±0.2 | 12.8±2.37 | 4476±18 | 4493±27 |
| **DNAJB1** | **Hsp40** | | 3.5±0.30 | 2.7±0.97 | | 1909±163 | 3008±85 |
| **HSPH1** | **Hsp105** | 2.8±0.02 | 6.1±0.33 | 12.4±2.1 | | 259±36 | 417±12.0 |

**TABLE 1. In vitro screening of chaperone/aSyn combinations on stimulated murine splenocytes in culture.** Assayed parameters were MCH-II$^+$/CD86$^+$ cells percentage as analysed by flow cytometry, and IFN-γ, IL-10, and IL-17 cytokine levels as determined by ELISA. Values correspond to the mean value ± SD of duplicate samples from one

| Chaperones | | IL-10 (pg/ml) | | | IL-17 (pg/ml) | | |
|---|---|---|---|---|---|---|---|
| *Protein name* | *Altern.* | *αSyn* | *Chap.* | *Comb.* | *αSyn* | *Chap.* | *Comb.* |
| **HSPA1A** | **Hsp70** | 10.1±0.12 | 21.0±1.3 | 27.1±0.6 | 15.3±0.1 | 19.8±0.8 | 23.7±4.2 |
| **HSPA8** | **Hsc70** | 24±8.0 | 322±18 | 293±20 | | 31.5±0.1 | 29.0±0.1 |
| **HSPA9** | **Grp75** | 56.4±1.5 | 113±3.5 | 99. 8±7.2 | 10.2±0.1 | 12.1±0. 6 | 13.5±0.8 |
| **HSP90B** | **Hsp90β** | | 27. 7±2.7 | 25.4±4.9 | | 16.4±0.8 | 20.4±1. 9 |
| **STUB1** | **CHIP** | | 91±10.7 | 103.5±4.9 | | 11.9±0.01 | 15.2±1.3 |
| **Cyp40** | | | 29±3.6 | 31.0±0.5 | | 14.2±0.4 | 15.1±0.3 |
| **FKBP1A** | **FKBP12** | 34±8.0 | 16.7±0.9 | 32±11.7 | 10.7±0.3 | 10.5±0.1 | 10.6±0.4 |
| **CypA** | | | 14.0±1. 6 | 18.4±0. 8 | | 14.4±1.0 | 14.9±0.7 |
| **PARK7** | **DJ- 1** | 21±7.9 | 24.5±1.9 | 20.5±3. 5 | | 10.5±0.01 | 12.7±0.1 |
| **PIN1** | **Pin1** | | 14.8±0.95 | 24.0±2.2 | 25.5±0. 9 | 21.9±1.5 | 22.1±1.2 |
| **HSPC4** | **Grp94/Gp96** | 10.0±1.1 | 61. 8±4.0 | 108.6±3.3 | 13.8±0.3 | 20.2±3.2 | 20.7±1.5 |
| **14-3-3γ** | | 11.9±0.1 | 8.8±0.05 | 12.8±2.0 | 14.9±2.8 | 17.6±5.0 | 20.1±3.3 |
| **14-3-3ε** | | 23±8.0 | 20.6±0.47 | 28.6±2.7 | 14.0±0.1 | 11.5±0.9 | 19.6±1.9 |
| **14-3-3t** | | 14±0.1 | 16.2±0.39 | 19. 5±0.1 | | 12.7±1.5 | 19.0±1.9 |
| **ST13** | **Hip** | 24.3±1.3 | 17.1±0.04 | 30±10.6 | 25.6±0.3 | 30.0±2.2 | 36.6±0.4 |
| **Apo-J** | **Clusterin** | 21.2±2.1 | 12.6±1.35 | 32.3±3.0 | | 22.3±0.7 | 22.4±0. 9 |
| **HSPB1** | **Hsp27** | 27±2.2 | 17.6±0.03 | 40±7.7 | 30.0±3.0 | 18.5±0.1 | 19.4±0.01 |
| **FKBP4** | **FKBP52/59** | | 277±3.5 | 347.9±2.5 | | 32.5±0.5 | 37.4±2.0 |
| **RAB11A** | | 11.0±0.1 | 115.4±1.9 | 103±7.1 | | 25.1±2.9 | 21.1±2.6 |
| **DNAJB1** | **Hsp40** | 27.2±2.2 | 28.9±1.2 | 53.8±3.3 | | 42±10.6 | 28.9±7.9 |
| **HSPH1** | **Hsp105** | | 61±4.5 | 99.0±2.5 | | 11.4±0.1 | 11.1±0.1 |

representative experiment. Altern., alternate; Chap., chaperone; Comb., combination. Previously identified chaperones based on results shown in **Fig. 1** are highlighted in grey. Selected chaperones are indicated in dark grey.

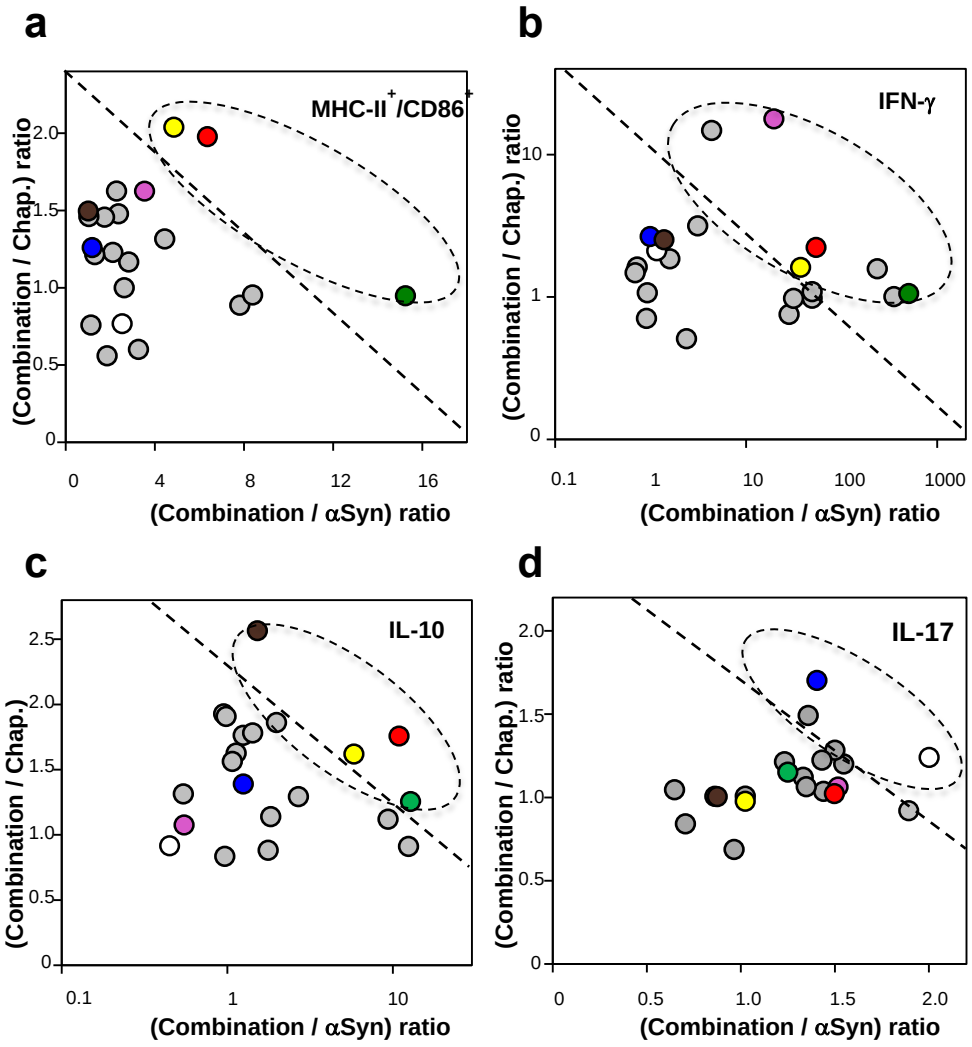



**Figure 1. In vitro screening of a set of 21 chaperone candidates and the corresponding chaperone/aSyn mixtures for selection of immunoactive combinations**. Murine splenocytes in culture at 1 x $10^6$ cells/well were stimulated with the various aSyn/chaperone combinations (at a 1:1 molar ratio, at the nanomolar level; see Materials and Methods for details), or with aSyn or the chaperones alone at the same concentrations used as controls and incubated at 37°C for 24 h. Splenocytes were then labeled for detection of CD86 and MCH-II surface markers and analysed by flow cytometry. The contents (percent) of double-positive cells for all the samples were determined (**Table 1**) and plotted as the ratios of values obtained for the different

combination/chaperone vs. the same combination/aSyn (**A**). The levels of IFN-γ (**B**), IL-10 (**C**), and IL-17 (**D**) cytokines in the supernatants of cultured splenoctyes were assayed (**Table 1**), and the values obtained were plotted in an analogous manner. As a first selection step, chaperones that fell within the top triangle in the plot were identified (**Table 1**). In a second round, previously identified chaperones that showed the highest measured values in combination with aSyn for $CD86^{+}$/$MHC\text{-}II^{+}$, IFN-γ, and IL-10, but not for IL-17, were singled out (Table 1). Based on this analysis, Grp94/Gp96 (red) and FKBP4/52 (green) chaperones were chosen for subsequent immunization studies. Other chaperones that showed extreme values in either plot are shown in color: Cyp40 (pink), 14-3-3ε (blue), Hsp105 (yellow), clusterin (brown), and Hsp90β (white).

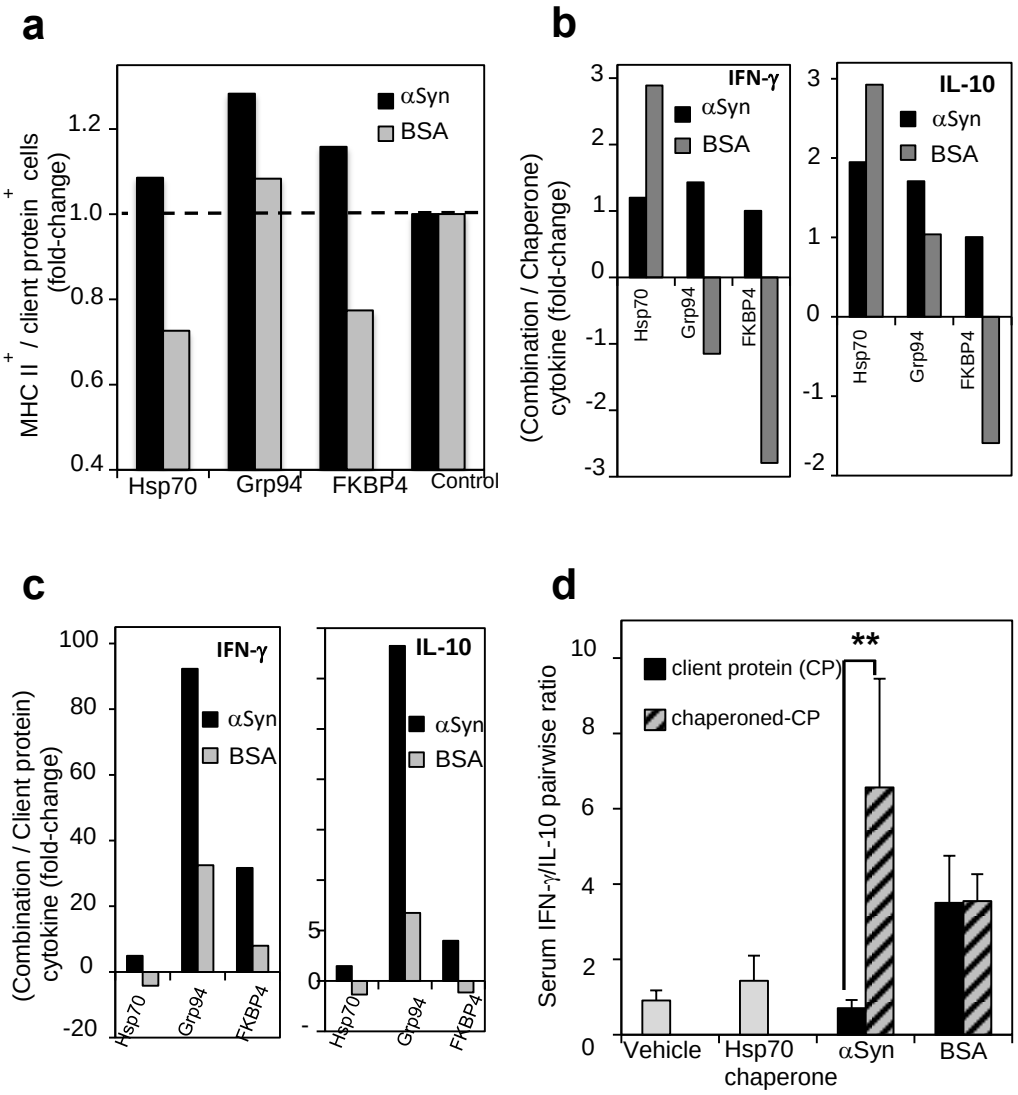
## Specific response elicited by chaperoned aSyn as compared with a nonrelevant client protein

To assess the specificity of the immune responses observed in the screening, we carried out a set of assays to compare the effect of Grp94, FKBP4, and Hsp70 (as a previously studied model chaperone) on the response elicited by aSyn or BSA as a nonrelevant client protein. For this purpose, we first quantified the uptake by cultured murine splenocytes of fluorescently labeled client protein (aSyn or BSA), alone or in the presence of either chaperone, by flow cytometry (**Supplemental Fig. 1A**). Our results show that although the proportion of MHC-II+/aSyn+ cells is higher in the presence of all 3 chaperones as compared with the non-chaperoned aSyn, the percentage of MHC-II+/BSA+ cells is lower or remains essentially unaltered in the presence of the chaperones, as compared with nonchaperoned BSA. Next, we quantified the cytokine release levels from stimulated splenocytes with the same protein mixtures after incubation for 24 h (**Supplemental Fig. 1B, C**). These results, which are in good agreement with those observed in the screening step, showed that all 3 chaperones affect the cytokine release profile elicited by aSyn and BSA in a differential manner and that the immune response is specific for

each client protein. Furthermore, we used Hsp70 as a previously studied chaperone to test the specificity of the immune response elicited by chaperoned aSyn or BSA in C57/BL6 mice, as a result of immunization with the different protein mixtures. According to the measured cytokine levels in serum from immunized mice, although immunization with Hsp70/aSyn caused a 7-fold higher IL-10:IFN-γ ratio as compared with aSyn, immunization with Hsp70/BSA did not change the IL-10:IFN-g ratio as compared with BSA, indicating that the chaperone affects the immune response differentially when comparing aSyn with a nonrelevant client protein (**Supplemental Fig. 1D**). Thus, our results indicate that the changes observed in the immune response generated in the presence of these chaperones depend on the identity of the accompanying client protein, and therefore that the immune regulation in subsequent experiments that combine these chaperones and aSyn can be anticipated to be aSyn dependent.

**Altered reactivity of splenocytes of mice immunized with Grp94- or FKBP4-chaperoned MaSyn**

To test and compare the immunogenic activity of both selected chaperones in vivo, we first applied an immunization protocol with each chaperone in conjunction with MaSyn. Mice were immunized with 1:1 molar ratios of chaperone:MaSyn preincubated mixtures or the corresponding controls, according to protocols 1 or 2 as described in Materials and Methods, as follows: with either Grp94, MaSyn (lower concentration), Grp94/MaSyn, or buffer alone/vehicle (protocol 1), or with FKBP4 MaSyn (higher concentration), FKBP4/MaSyn, or buffer alone/vehicle (protocol 2).One week after the booster injection, the mice were killed, and their spleens and whole blood were extracted for further analyses.




**Suppl. Figure 1. Assessing the specificity of the immune response elicited by chaperoned aSyn.** Uptake of AF647-labeled client proteins (CP) aSyn and BSA by mouse splenocytes after 1 hr incubation in the presence or absence of Hsp70, Grp94 or FKBP4 chaperones (1:1 molar ratio), as measured by flow cytometry (a). Shown values are the fold-change of cells with MHC-II-positive signal and internalized proteins (MHC-II+/CP+ cells) as compared to non-chaperoned CP, for each aSyn and BSA. Cytokine release profiles (IFN-γ and IL-10) of cultured murine splenocytes after a 24 h-incubation with aSyn or BSA in the presence or absence of Hsp70, Grp94 or FKBP4 chaperones (1:1

molar ratio) (b). Pre-incubation mixtures with aSyn or BSA and the chaperones (or buffer control) were prepared in all cases. Shown values correspond to the ratio of 'chaperone-CP mixture' over the 'chaperone', signals (left panels) and to the 'chaperone-CP mixture' over the 'CP', signals (right panels). Results shown are representative of two independent experiments. IFN-γ levels for non-chaperoned aSyn and BSA were 4.5±1.8 pg/ml and 7.4±1.6 pg/ml , respectively. IL-10 levels were 5.0±2.7 pg/ml and 6.4±4.3 pg/ml.

Serum cytokine profile of C57/BL6 mice immunized with Hsp70/γSyn or Hsp70/BSA combinations at a 1:1 molar ratio (and the corresponding controls with either protein alone or buffer/vehicle) by using Hsp70 chaperone as a model. Immunizations were performed in the absence of added adjuvant, as previously described in the Materials and Methods section (c). Shown values are means ± S.E.M. (N = 5 for 'vehicle', 'Hsp70', 'aSyn' and 'Hsp70/aSyn' groups; N=3 for 'BSA' and 'Hsp70/BSA' groups). Asterisks indicate statistically significant differences between a particular group and the 'vehicle' group. ** P<0.01.

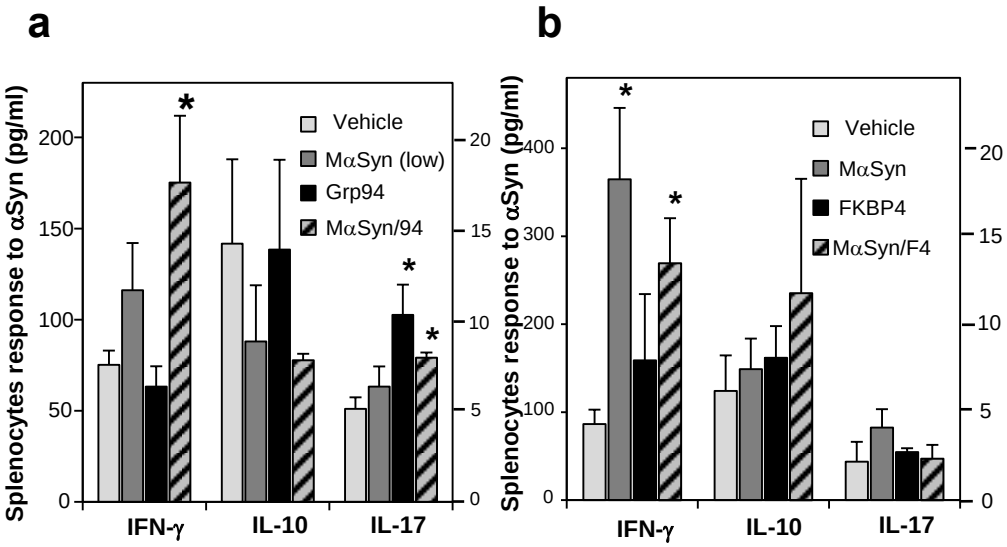
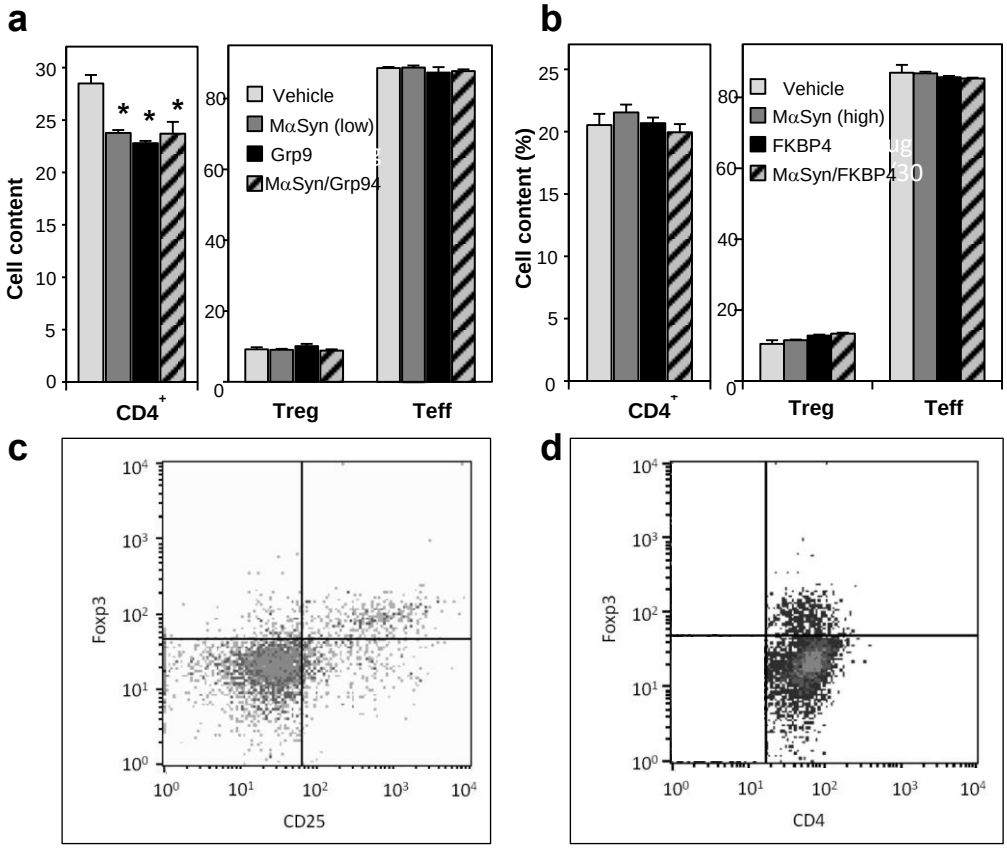



**Figure 2. Cytokine secretion profiles from aSyn-stimulated splenocytes of mice immunized with Grp94- (A) or FKBP4-(B) chaperoned MaSyn**. IFN-γ (*y* axis), IL-10 (*z* axis), and IL-17 (*z* axis) levels were measured by ELISA in the supernatants of cultured splenocytes from immunized mice, 24 h after treatment with aSyn (20 μg/ml). Values are means ± SEM (n = 5). Asterisks indicate statistically significant differences between a particular group and the vehicle group. *P < 0.05.

To explore possible aSyn-specific hyper-/hypoimmune responses, we measured cytokine release profiles of cultured splenocytes isolated from immunized mice. To this end, cultured cells from the different experimental groups were pulsed with 20μg/ml aSyn (or with medium alone as a control). Levels of IFN-γ and IL-17 (which are involved in the helper T-cell–mediated $T_h1/T_h17$ responses) as well as IL-10 were measured in the culture supernatants collected after 24 h of incubation (**Fig. 2A, B**). The data obtained showed an increase in the secretion of IFN-γ as a result of immunization with MaSyn/Grp94 (175 ± 37 vs. 75.2 ± 7.9 pg/ml) and of IL-17 as a result of immunization with Grp94 (10.3 ± 1.7 pg/ml) and with the MaSyn/ Grp94 combination (7.9 ± 0.3 pg/ml) as compared with the control (5.1 ± 0.6 pg/ml), which indicates a higher $T_h1/T_h17$ response to aSyn challenge in the case of MaSyn/Grp94 (**Fig. 2A**). On the other hand, splenocytes from mice immunized with the highest concentration of MaSyn and with the MaSyn/FKBP4 combination produced higher IFN-γ levels (365 ± 81 and 269 ± 51 pg/ml, respectively) than the control (87 ± 16.3 pg/ml) when stimulatedin vitro with aSyn (**Fig. 2B**), indicative of a higher $T_h1$ aSyn-specific response.

Because of the key role of $CD4^+$Tcells in the development of synucleinopathies and other misfolding diseases associated with chronic neuro inflammation, we analysed the$CD4^+$ T-cell population within splenocytes by flow cytometry, which allowed the detection of lower levels of $CD4^+$ cells in the groups immunized with the lowest concentration of MaSyn (23.8 ± 0.3%), with Grp94 (22.8 ± 0.2%), and with the MaSyn/Grp94 combination (23.7 ± 1.1%) relative to the control group (28.5 ± 0.8%) (**Supplemental Fig. 2A**). However, no significant alterations in the $CD4^+$ T-cell population were observed for the groups immunized with the highest concentration of MaSyn, with FKBP4, or with the MaSyn/FKBP4 combination, nor in the $CD4^+CD25^+Foxp3^+$ ($T_{reg}$) and CD4+$Foxp3^-$

($T_{eff}$) cell population levels for either experimental group (**Supplemental Fig. 2A**). Thus, we observed alterations in the cytokine response of the splenocytes to a challenge with aSyn that are apparently not related to changes in the $T_{reg}$ or $T_{eff}$ cellular contents.




**Suppl. Figure 2. CD4+, Treg and Teff cell populations in splenocytes from mice immunized with Grp94- or FKBP4- chaperoned MaSyn**. Percentage of CD4+ cells (from total splenocytes, left), and Treg (CD4+CD25+Foxp3+) and Teff (CD4+Foxp3-) cells (right), in isolated splenocytes from mice immunized with MaSyn (lower dose), Grp94, or the MaSyn/Grp94 combination (a), and with MaSyn (higher dose), FKBP4, or the MαSyn/FKBP4 combination (b). The Treg (c) and Teff (d) cell populations were calculated as the $CD4^+CD25^+Foxp3^+$ stained cells and the $CD4^+Foxp3^-$ stained cells, respectively, among the $CD4^+$ lymphocyte population. Represented values are mean ±

S.E.M. (N = 5). Asterisks correspond to statistically significant differences between one particular group and the 'vehicle' group. *P<0.05, ** P<0.01. Plots are from one representative mouse sample for each analysis.

**Immunization with Grp94- or FKBP4-chaperoned MaSyn produces a differential antigen-specific antibody response**

To analyse the antigen-specific humoral response in the different experimental groups as a result of immunization, total and aSyn-specific IgM and IgG levels were assayed by ELISA in serum obtained 1 wk after the booster injection (**Fig. 3 and Supplemental Fig. 3**). Although no significant changes in absolute IgM or IgG levels were observed for either experimental group (**Supplemental Fig. 3A, B**), a significant increase in the anti-aSyn/total IgG content was produced by immunization with the MaSyn/Grp94 combination (28.7 ± 3.4 vs. 18.6 ± 2.9 AU) (**Fig. 3A and Supplemental Fig. 3C**). On the other hand, the slight increments in anti-aSyn/total IgM contents seen for groups immunized with the highest concentration of MaSyn and with FKBP4 (15.6 ± 1.6 and 15.3 ± 1.0 AU, respectively) as compared with the control (11.2 ± 0.7AU) was suppressed by the MaSyn/FKBP4 combination (14.4 ± 1.4 AU) (**Fig. 3B**), although no differences were observed in the aSyn-specific IgG contents in this group (**Fig. 3B and Supplemental Fig. 3D**). Furthermore, no significant shifts in the aSyn-specific IgG isotype ratios were detected for either experimental group (**Fig. 3C, D**). Overall, our results indicated that immunization with Grp94/MaSyn produced an increase in levels of anti-aSyn IgG antibodies with unaltered IgG isotype profile.

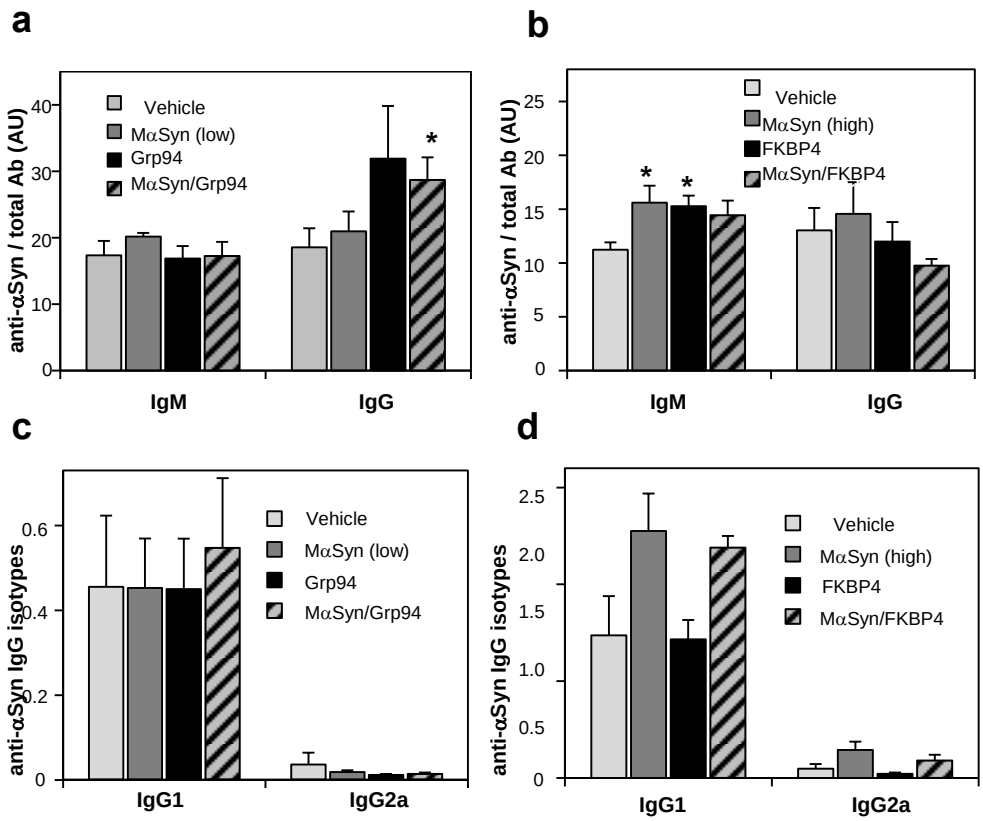
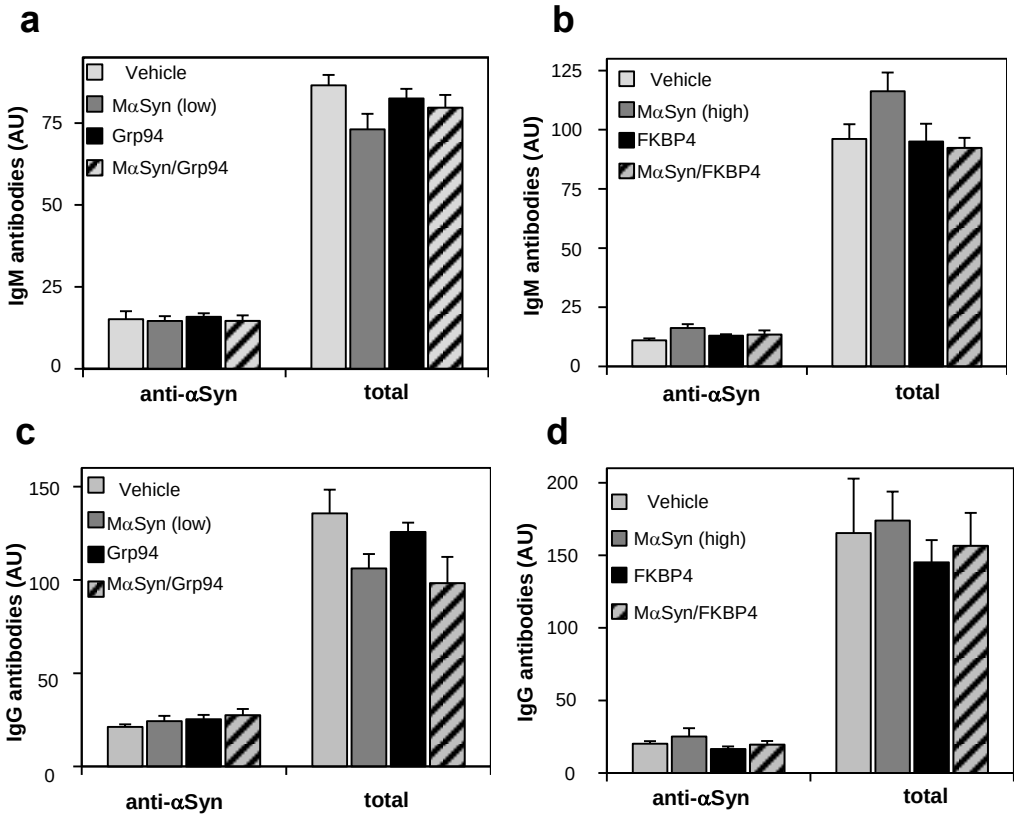



**Figure 3. aSyn-specific humoral response in mice immunized with Grp94- or FKBP4-chaperoned MaSyn.** Relative anti-aSyn IgM and IgG levels in serum from mice immunized with MaSyn (lower dose), Grp94, or MaSyn/Grp94 **(A)** and with MaSyn (higher dose), FKBP4, or MaSyn/FKBP4 **(B)**. The relative content of specific anti-aSyn IgM and anti-aSyn IgG antibodies were calculated by dividing the levels of anti-aSyn IgM or anti-aSyn IgG antibodies by the total IgM or IgG antibody levels, respectively, for each mouse. Data are shown for anti-aSyn IgG1 and IgG2a, levels in serum after immunization with MaSyn (lower dose), Grp94, or MaSyn/Grp94 **(C)** and with MaSyn (higher dose), FKBP4, or MaSyn/FKBP4 **(D)**. AU values relate only to the internal control (vehicle). The values shown are means ± SEM (n = 5). Asterisks indicate statistically significant differences between a particular group and the vehicle group. *P < 0.05.




**Suppl. Figure 3. Humoral response in mice immunized with Grp94- or FKBP4-chaperoned MaSyn**. Anti-aSyn IgM and total IgM levels in serum from mice immunized with MaSyn (lower dose), Grp94, or MaSyn/Grp94 **(a)**, and with MaSyn (higher dose), FKBP4, or MaSyn/FKBP4 **(b)**, were determined by ELISA. Anti-aSyn IgG and total IgG levels in serum from mice immunized with MaSyn (lower dose), Grp94, or MaSyn/Grp94 **(c)**, and with MaSyn (higher dose), FKBP4, or MaSyn/FKBP4 **(d)**. AU: arbitrary units; values relate only to the internal control (vehicle). Represented values are mean ± S.E.M. (N = 5). Asterisks correspond to statistically significant differences between one particular group and the 'vehicle' group. *P<0.05, **P<0.01. AU: arbitrary units.

**Grp94- and FKBP4-chaperoned MaSyn produce differential immunologic phenotypes in the peripheral immunity**

To characterize further and compare the peripheral immune response elicited by immunization with both chaperone/aSyn combinations, we analysed the IgG1/IgG2 profile of the humoral response by measuring the IgG1 and IgG2 total antibody levels, as well as the calculated pairwise IgG1:IgG2a ratio for each experimental animal (**Fig. 4A, B**). Interestingly, the aSyn/Grp94 combination produced an ~2.5-fold increase in the IgG1:IgG2a ratio (26.8 ± 3.7 vs. 12.3 ± 2.4 AU), suggesting a $T_h2$ skewing of the peripheral response (**Fig. 4A, inset**). Given that the relative levels of IFN-γ and IL-10 are believed to be important in determining the balance between $T_h1/T_h2$ cells (Katsikis et al. 1995)—which in turn determines the direction of activation of the cell mediated or the humoral immune responses—we measured the levels of these 2 key cytokines in serum and calculated the pairwise IL-10:IFN-γ ratio for each mouse (**Fig. 4C, D**). Interestingly, although no changes were detected for the Grp94 and MaSyn/Grp94 groups (**Fig. 4C**), some changes were observed for the highest MaSyn and MaSyn/FKBP4 experimental groups, namely an increase in the IFN-γ levels (36.3 ± 2.6 and 39.2 ± 8.1 pg/ml, respectively) relative to the control group (26.5 ± 0.5 pg/ml), and concomitant reductions in the pairwise IL-10:IFN-γ ratios (1.05 ± 0.07 and 0.89 ± 0.1) as compared with the control group (1.4 ± 0.16), suggesting some degree of $T_h1$ polarization of the general response (**Fig. 4D**).

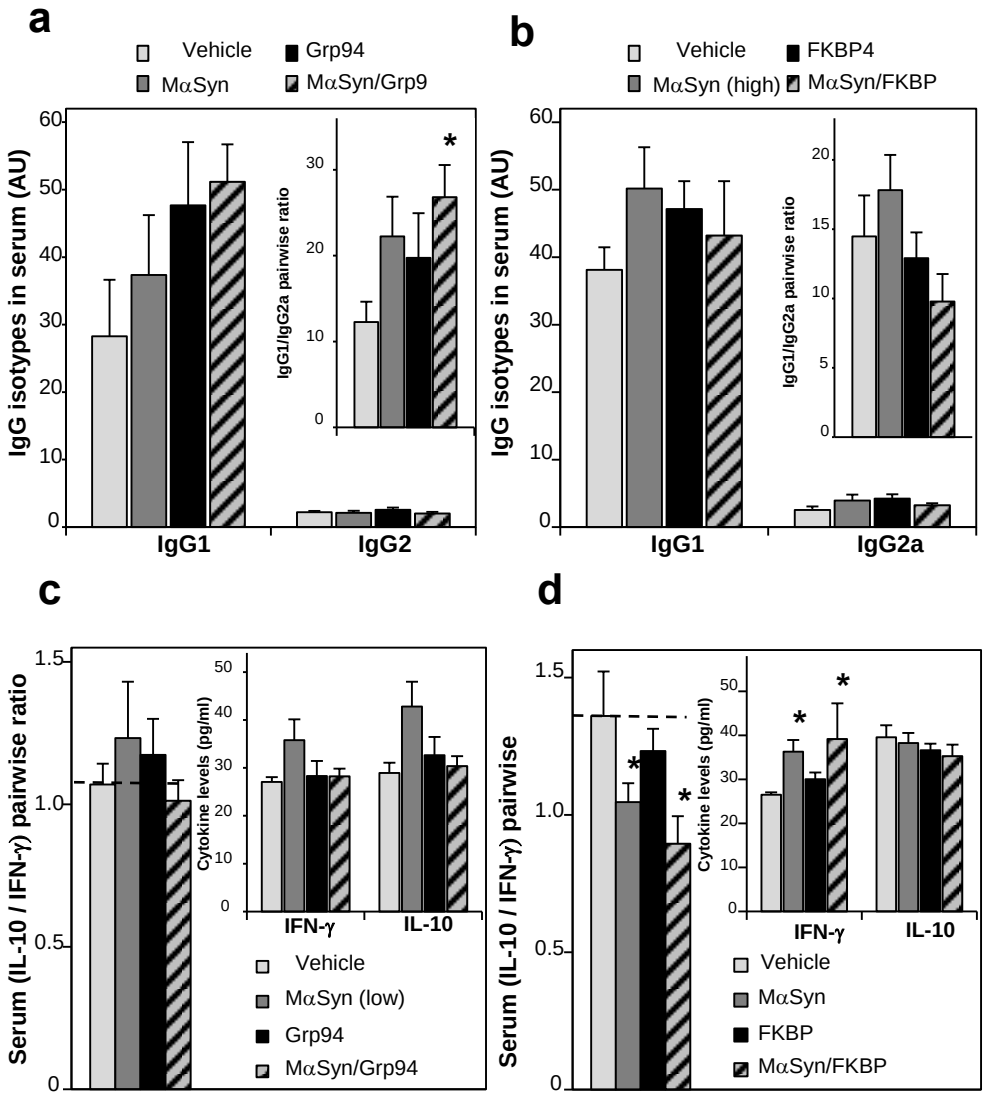



**Figure 4. Th1/Th2 profiling in serum from mice immunized with Grp94- or FKBP4-chaperoned MaSyn.** Total IgG1 and IgG2a antibody levels in serum from mice immunized with MaSyn (lower dose), Grp94, or MaSyn/Grp94 **(A)** and with MaSyn (higher dose), FKBP4, or MaSyn/FKBP4 **(B)** were determined by ELISA. Pairwise IgG1:IgG2a ratios in each case **(A, B, inset)** were calculated by dividing the measured IgG1 level by the measured IgG2a level for each mouse. AU values relate to the internal control (vehicle). IFN-γ and IL-10 levels were measured in sera from immunized mice by ELISA **(C, D, inset)**. Pairwise IL-10:IFN-γ ratios were calculated by dividing the measured

IL-10 cytokine level by the measured IFN-γ cytokine level for each mouse for Grp94 and FKBP4, experimental groups **(C, D)**. The values shown are means ± SEM (n = 5). Asterisks indicate statistically significant differences between a particular group and the vehicle group. *P , 0.05.

**Grp94- and FKBP4-chaperoned OaSyn result in differential Th2-like responses**

As the aggregated form of aSyn is believed to be crucially involved in the pathogenesis of PD and other synucleinopathies, we decided also to investigate the response generated by immunizing mice with combinations of Grp94 or FKBP52 chaperones and OaSyn. To this end, we prepared aSyn oligomers (see Materials and Methods) and used them to immunize mice in the absence of added adjuvant, with the aSyn/chaperone combinations or the proteins alone (see immunization protocol 3 in Materials and Methods), after which analysis of isolated splenocytes from immunized mice by flow cytometry was carried out. In agreement with the findings of the previous experiments, a slight reduction in $CD4^+$ cell content was observed for the Grp94 group(21.4 ± 0.9% vs. 23.0 ± 0.4% for the control), in addition to the OaSyn/Grp94 group (21.5 ± 0.6%), even though the differences were not statistically significant. On the other hand, both the $T_{reg}$ and $T_{eff}$ cell content remained unchanged in the various groups as compared with the control (data not shown). As before, splenocytes were cultured and stimulated in vitro with 20 μg/ml aSyn (or with medium alone), and after 24 h, the culture supernatants were recovered and used for IFN-γ, IL-10, and IL-17 cytokine assays using ELISA (**Fig. 5**). Remarkably, a clear reduction of secreted IFN-γ levels (40 ± 23 pg/ml) as compared with the control group (168 ± 19 pg/ml) was measured for mice immunized with OaSyn/Grp94, suggesting a reduction in the $T_h1$ response linked to aSyn stimulation. On the other hand, no significant changes were observed for either IL-10 or IL-17 levels as compared with the control group (**Fig. 5**).

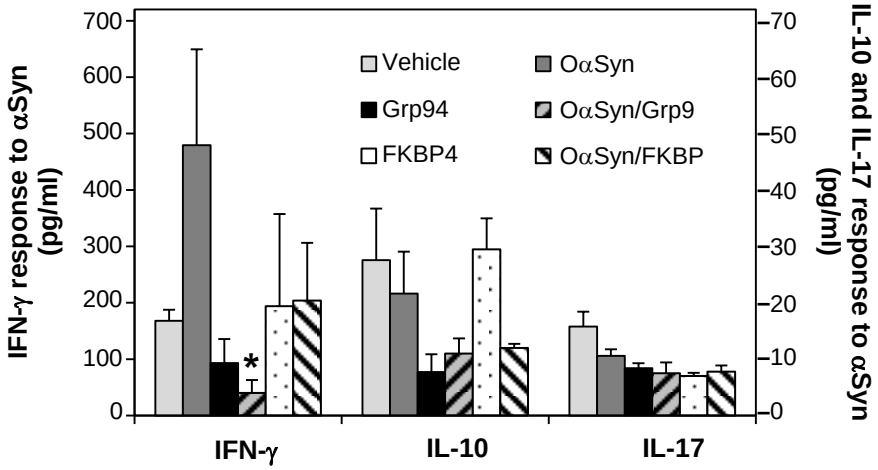



**Figure 5. Cytokine secretion profile from aSyn-stimulated splenocytes from mice immunized with Grp94- or FKBP4- chaperoned OaSyn**. IFN-γ (*y* axis), IL-10 (*z* axis), and IL-17 (*z* axis), levels were measured by ELISA in supernatants from cultured splenocytes from immunized mice 24 h after treatment with aSyn (20 μg/ml). The values shown are means ± SEM (n = 5). Asterisks indicate statistically significant differences between a particular group and the vehicle group. *P < 0.05.

We next assayed the humoral response elicited by immunization with OaSyn and chaperones for the different mice groups. The results showed that OaSyn produced approximately 2-fold higher levels, as compared with the vehicle, of both absolute (23.9 ± 2.3 vs. 10.3 ± 1.5 AU) and relative (53.8 ± 5.5 vs. 27.5 ± 4.7 AU) anti-aSyn IgM antibodies (**Fig. 6A**). This increase was potentiated by Grp94 within the OaSyn/Grp94 combination (34.9 ± 6.3 and 71.9 ± 5.7 AU, respectively), despite this effect not being observed with the chaperone alone (**Fig. 6A**). Moreover, a more significant increase was also observed in the anti-aSyn IgG levels for the OaSyn/Grp94 group, with an approximately 4-fold increase in absolute levels (35.5 ± 11.8 vs. 8.4 ± 0.6 AU for the control group), and an approximately 6-fold increment in relative anti-aSyn IgG (60.5 ± 19.5 vs.

16.6 ± 3.3 AU for the control group) (**Fig. 6B**). Immunization with OaSyn/FKBP4 caused a similar albeit milder effect on the anti-aSyn IgM and IgG response. Interestingly, such higher aSyn-specific IgG titers were largely caused by an increase in IgG1 antibodies rather than in IgG2a antibodies (**Fig. 6C, D**), although an increase trend for the IgG2a isotype was seen for the OaSyn/FKBP4 experimental group. These observations suggest that immunization with OaSyn elicits an aSyn-specific $T_h2$ type of humoral response, which is potentiated by its combination with theGrp94 chaperone. Remarkably, this phenotype was well reproduced in MPTP-treated (PD model) mice that had previously received splenocytes from immunized animals by adoptive transfer (AT), in which higher IgG1 and IgG2a titers of anti-aSyn antibodies were specifically measured for the OaSyn/Grp94 and OaSyn/FKBP4 groups, respectively (**Fig. 6E, F**). Of note, such an effect was detected 4 mo after the AT had been performed. These results strongly suggest that the effects observed upon immunization with chaperoned aSyn are robust and stable in time.

Finally, to analyze the peripheral response generated by the OaSyn-based immunization protocol, we assayed the total IgG1/IgG2a levels in serum (**Fig. 7A**). Despite higher levels of IgG1measured for the Grp94 group (86.0 ± 11.3 vs. 45.8 ± 8.8 AU), no significant changes in the pairwise IgG1:IgG2a ratios were detected in either experimental group compared with the control. On the other hand, based on the serum levels of IFN-γ and IL-10 cytokines measured by ELISA, the calculated pairwise serum IL-10:IFN-γ ratio showed approximately 2- to 3-fold increases in the OaSyn/Grp94 (1.15 ± 0.09) and OaSyn/FKBP4 (2.0 ± 0.47) groups as compared with the control (0.71 ± 0.04) (**Fig. 7B**). This result indicates a shift toward a $T_h2$-type phenotype caused by immunization with FKBP4- and Grp94-chaperoned OaSyn.

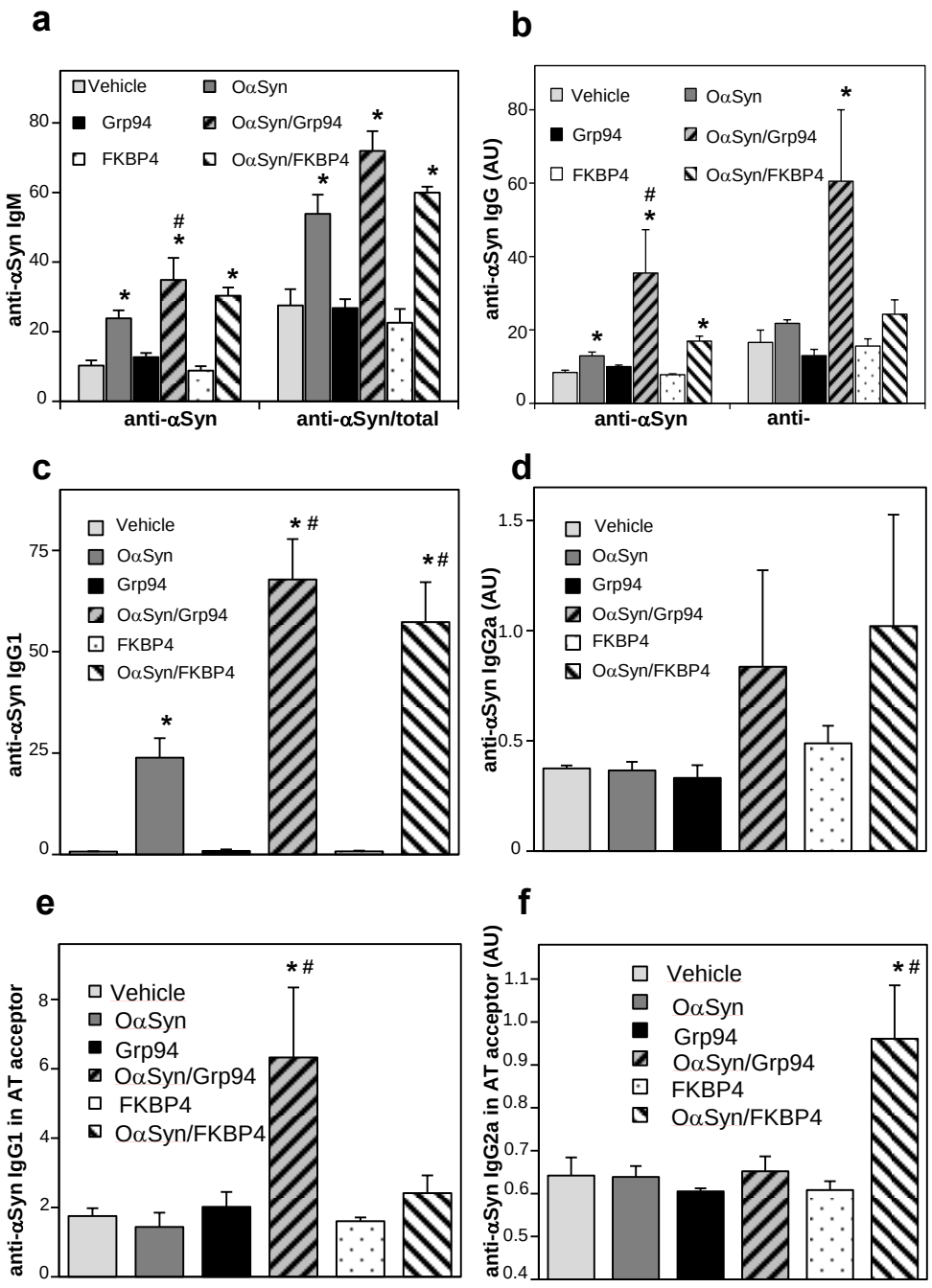



**Figure 6. aSyn-specific humoral response in mice immunized with Grp94- or FKBP4-chaperoned OaSyn and in adoptively transferred mice**. Absolute and relative anti-aSyn

IgM **(A)** and IgG **(B)** levels in serum from mice immunized with OaSyn, Grp94, OaSyn/Grp94, FKBP4, or OaSyn/FKBP4. The relative contents of specific anti-aSyn IgM and IgG antibodies were calculated by dividing the levels of anti-aSyn IgM or IgG antibodies by the total corresponding antibody levels for each mouse. Anti-aSyn IgG1 **(C)** and IgG2a **(D)** levels in serum of mice immunized with OaSyn, Grp94, OaSyn/Grp94, FKBP4, or OaSyn/FKBP4. Anti-aSyn IgG1 **(E)** and IgG2a **(F)** levels in serum of (PD model) MPTP-treated mice 5 mo after receiving splenocytes through AT from donor mice immunized with OaSyn, Grp94, OaSyn/Grp94, FKBP4, or OaSyn/FKBP4. AU values relate only to the internal control (vehicle). The values shown are means ± SEM (n = 5). Asterisks indicate statistically significant differences between a particular group and the vehicle group. Hash signs indicate statistically significant differences between a given chaperone/aSyn combination and the corresponding chaperone and aSyn groups. */# P< 0.05.

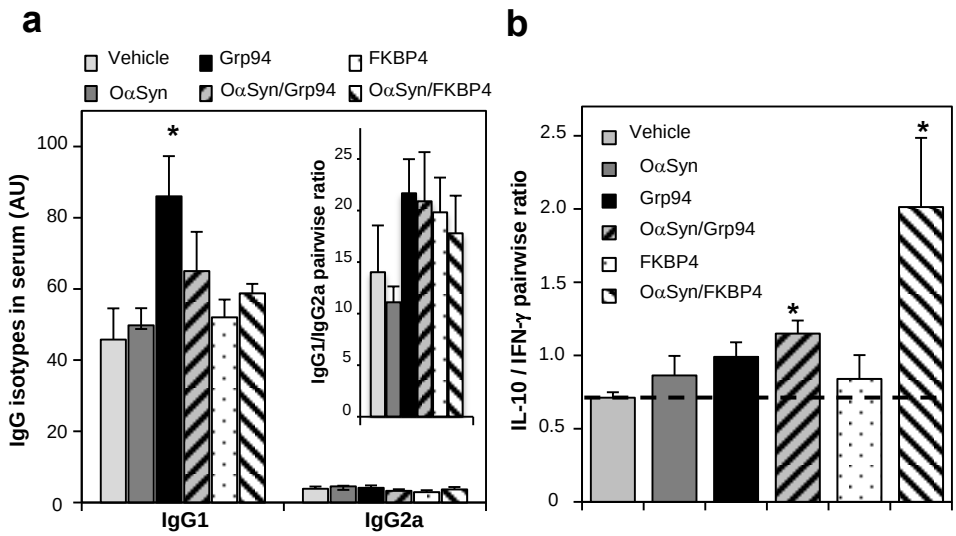



**Figure 7. $T_h1/T_h2$ profiling in serum from mice immunized with Grp94- or FKBP4-chaperoned OaSyn**. Total IgG1 and IgG2a antibody levels in sera from mice immunized with OaSyn, Grp94, OaSyn/Grp94 FKBP4, or OaSyn/FKBP4 were determined by ELISA **(A)**. AU values relate only to the internal control (vehicle). Pairwise IgG1:IgG2a ratios **(A, inset)** were calculated by dividing the measured IgG1 level by the measured IgG2a level for each mouse. IFN-γ and IL-10 levels were measured in sera from immunized

mice by ELISA **(Supplemental Fig. 4)**. Pairwise IL-10:IFN-γ ratios were calculated by dividing the measured IL-10 cytokine level by the measured IFN-γ cytokine level for each mouse **(B)**. The values shown are means ± SEM (n = 5). Asterisks indicate statistically significant differences between a particular group and the vehicle group. *P< 0.05.

## 5. Discussion

In addition to the traditional functions of molecular chaperones in assisting polypeptide folding and preventing protein misfolding and aggregation, a diversity of HSP-mediated immune responses of the innate and adaptive system have recently been described for certain HSPs (Binder 2014; Srivastava 2002; Quintana & Cohen 2011). Such adjuvant activities, also displayed by exogenously administered HSPs, have already been used in immunotherapy of cancer and infectious and autoimmune diseases (Binder 2014; Srivastava 2005). As a result of exploiting the immunofunctional properties and the chaperoning activity of human Hsp70, we found that immunization with the aSyn/Hsp70 complex generated an immunomodulatory phenotype in the peripheral system together with limited aSyn-specific antibody/humoral response (Labrador-Garrido et al. 2014). In the present work, as an approach to explore the immunomodulatory capabilities of the chaperome in relation to a misfolding protein, and to select suitable chaperones to accompany aSyn for immunotherapy of PD, we started out by screening a large set of preselected chaperone candidates. For this purpose, we designed and set up an in vitro screening procedure with cultured murine splenocytes and tested the immune response elicited by the chaperone candidates in combination with aSyn. Remarkably, differential immunomodulatory profiles were revealed by the screening, indicating that several chaperones including Hsp105, Hsp40, Cyp40, Pin1, Grp94/Gp96, FKBP4/52, Rab11A, and Apo-J/clusterin, among others, could modify significantly the aSyn-elicited immune response.

Based on the screening results, we selected 2 chaperones— Grp94/Gp96 and FKBP4/52—as good candidates for subsequent immunization studies. FKBP4/52 is a member of the immunophilin protein family, whose name reflects a high binding affinity toward the natural immunosuppressant drug FK506. Although the established physiologic function of FKBP4/52 is that of a cytosolic chaperone with peptidyl-prolyl cis/trans isomerase (rotamase) activity (Barik 2006), 2 studies have reported its participation in the regulation of lymphocyte activity via its isomerase activity (Nath & Isakov 2015; Mamane et al. 2000; Chambraud et al. 1996). Pathologically, FKBP4/52 has been linked with neurodegeneration and was shown to interact with tau and aSyn proteins (Gerard et al. 2011; Deleersnijder et al. 2011; Giustiniani et al. 2015). The other selected chaperone, Grp94/Gp96, has a typically endoplasmic location and can be found in the extracellular space in situations of stress and exposure to immunologic danger signals. Following pioneering work by Srivastava et al. (Srivastava et al. 1986), a number of studies have demonstrated that its immunologic specificity originates from the bound peptides, and several clinical trials of immunotherapy in cancer patients based on Grp94/Gp96 preparations are currently under way (Randazzo et al. 2012). In addition, altered levels of Grp94/Gp96 have been linked to neurodegeneration (Kurzawa-Akanbi et al. 2012; Di Domenico et al. 2010), and the chaperone has been shown to bind to intracellular aSyn in a cellular model of PD (Jin et al. 2007).

The FKBP4/52 and Grp94/Gp96 chaperones selected in this way were used to prepare MaSyn or OaSyn/chaperone mixtures, and the corresponding controls of chaperones and aSyn proteins alone or vehicle were chosen to immunize C57BL/6 mice in the absence of added adjuvant. Our results from aSyn-challenged splenocytes showed that immunization with MaSyn/Grp94 promoted a $T_h1/T_h17$ anti-aSyn cellular response, whereas MaSyn/FKBP4 elicited a higher $T_h1$ response to aSyn stimulation. Interestingly enough, immunization with OaSyn/Grp94 produced an attenuation of the$T_h1$ cellular immunity, that is, a higher $T_h2/T_h1$ response to aSyn stimulation, as compared with the control.

In additional experiments, analysis of the anti-aSyn humoral response developed in mice immunized with MaSyn showed that only the MaSyn/Grp94 combination produced an increase in anti-aSyn antibody titers, and it did so without modifying the IgG isotype contents. On the other hand, immunization of mice with OaSyn by itself increased the IgM and IgG anti-aSyn antibody levels, and such an effect was potentiated by Grp94 and, to a lesser extent, by FKBP4. Interestingly, such an increase in anti-aSyn IgG titers by Grp94 was found to be produced exclusively by higher anti-aSyn IgG1 isotype levels, once again indicating a $T_h2$-skewing of the antigen-specific humoral immunity by OaSyn/Grp94. Conversely, an increase trend in anti-aSyn IgG2a antibodies was seen in mice immunized with OaSyn/FKBP4, suggesting a $T_h1$ skewing of this response. Remarkably, this pattern was reproduced in a mouse model of PD months after receiving splenocytes by AT from immunized donors, which supports the notion that these long-term changes generated by immunization with chaperoned aSyn are robust and physiologically relevant.

The final set of experiments, assessment of the IgG1:IgG2a ratio of total serum antibody titers, revealed that immunization with the MaSyn/Grp94 combination increased this ratio and therefore indicates a skewing of the general peripheral immunity toward a $T_h2$-type phenotype, despite the observation of a lack of significant change in the IL-10/IFN-γ serum levels. In contrast, immunization with the MaSyn/FKBP4 combination produced unaltered total IgG1 and IgG2a levels but reduced the IL-10:IFN-γ ratio in the serum, consistent with a skewing of the general peripheral immunity toward a $T_h1$-type phenotype. Remarkably, this effect was reversed when OaSyn was used in the immunization protocol in the OaSyn/FKBP4 combination, which generated a considerable increase IL-10:IFN-γ ratio in serum, indicating a skewing of the general peripheral immunity toward a $T_h2$-type phenotype. Interestingly, our results suggest that immunization with Grp94/Gp96- and FKBP4/52-chaperoned aSyn produced responses that dissociate the aSyn-specific immunity from the general immunologic phenotype.

Taken together, our work reveals that a variety of molecular chaperones display an array of immunomodulatory activities in vitro. Interestingly, a recent study has shown some of these chaperones, or their close homologs, to be part of a chaperome subnetwork critically involved in ageing and neurodegenerative disease, including PD (Brehme et al. 2014). Indeed, although several studies have focused on its essential role as the guardian of proteostasis, the potential of the chaperome as a modulator of the immune response has not previously been addressed. Even though there is still a clear need to understand further the role of $T_h1/T_h2/T_h17$ imbalances in the development of PD, our results show that in addition to Hsp70, Grp94/Gp96 and FKBP4/52 can be used effectively to chaperone aSyn and modulate the immune response in diverse ways, depending both on the chaperone involved and on the aggregation state of the aSyn protein. Based on these first observations, we propose that such immunochaperones could potentially act as highly versatile tools to redirect the immune response in misfolding diseases at the appropriate time of disease progression, both for basic studies of the immune profiles associated with neurodegeneration and to serve as a novel immunotherapeutic strategy for PD and perhaps other related amyloid disorders.

## 6. Acknowledgements

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# CONCLUSIONS

3. Immunization of healthy mice with aSyn/Hsp70 produces a shift in the peripheral immune system from a pro-inflammatory profile towards an immunomodulatory profile.

4. Several members of the chaperome, including Hsp105, Hsp40, Cyp40, Pin1, Grp94/Gp96, FKBP4/52, Rab11A, and Apo-J/clusterin, significantly modify the aSyn-elicited immune response of stimulated lymphocytes, in vitro.

5. Immunization with monomeric aS/Grp94 promotes a Th1/Th17 anti-aSyn phenotype coupled to increased antigen-specific humoral response, while generating a skewing towards a Th2 peripheral, general immune state.

6. Immunization with monomeric aS/FKBP4 promotes a Th1 anti-α-synuclein response accompanied by a Th1 peripheral, general immune state.

7. Immunization with oligomeric aS/Grp94 promotes a skewing towards a Th2 anti-α-synuclein phenotype together with increased antigen-specific humoral response, while generating a skewing towards a Th2 peripheral, general immune state.

8. Immunization with oligomeric aS/FKBP4 promotes a skewing to a Th1 anti-α-synuclein phenotype accompanied by a Th2 peripheral, general immune profile.

9. Immunization with Grp94/Gp96- and FKBP4/52-chaperoned α-synuclein drives the dissociation, in the peripheral system, of the α-synuclein-specific immune responses from the general immune phenotype.

10. Grp94/Gp96 and FKBP4/52 can be used effectively to chaperone aSyn and modulate the immune response, depending both on the chaperone involved and on the aggregation state of the α-synuclein protein.

11. Immunization via adoptive transfer of splenocytes from mice vaccinated with monomeric aS/Grp94 promotes a Th1-skewed anti-α-synuclein phenotype, while generating an immunomodulatory/Th2 peripheral general immune state in recipient PD model mice.

12. Direct vaccination with monomeric aS/Grp94 promotes a Th1/Th17 anti-α-synuclein phenotype coupled to increased antigen-specific humoral response, while generating a skewing towards a Th2 peripheral, general immune state in PD model mice.

## CHAPTER 3: Book Chapters

# Alpha-Synuclein and the Immune Response in Parkinson's Disease

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# The Hsp70 Chaperone System in Parkinson's Disease

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# Alpha-Synuclein and the Immune Response in Parkinson's Disease

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## 1. Introduction

In the last few years, it has become evident that the immunological component is of central importance in Parkinson's Disease (PD) pathogenesis and progression. This can also certainly be said about the prominent role that the protein α-synuclein (aSyn) is currently believed to play in the pathobiology of this neurodegenerative disorder. Moreover, the multiple mechanisms through which aSyn might be affecting the immune system appear not to be just a consequence of disease progression, but to actively contribute to the delicate balance between neuroprotection and neurotoxicity that ultimately underlies a given stage of disease.

PD is a proteinopathy, whose pathological hallmark is the presence of deposits of aggregated aSyn in intracellular fibrillar inclusions in neurons of the substantia nigra pars compacta (SN) of the brain, known as Lewy bodies (LB) (Spillantini *et al.*, 1998; Croisier *et al.*, 2005), and the loss of dopaminergic neurons (Braak *et al.*, 2003). Three missense mutations, A53T, A30P and E46K, as well as multiple copies of the wild-type (Wt) aSyn gene, are linked to early-onset, familial PD (Polymeropoulos *et al.*, 1997; Krüger *et al.*, 1998; Zarranz *et al.*, 2004; Gasser, 2005). Given that aSyn is the major component of LB in both familial and sporadic PD cases (Spillantini *et al.*, 1998), aSyn is considered a

critical factor in PD aetiology. Currently, the cellular and molecular mechanisms underlying the pathological actions of aSyn are poorly understood, and the factors contributing to sporadic PD, representing the vast majority of PD cases, are not known.

aSyn, together with β- and γ-synucleins, belong to the family of synucleins, a group of closely related, brain-enriched proteins. This 140 aa-residue protein is largely located in neuronal presynaptic terminals (Kim, Seo and Suh, 2004) and in the nucleus (Yu *et al.*, 2007). In particular, it is found in the neocortex, hippocampus and SN (Kim, Seo and Suh, 2004), and in other brain regions, as well as within astrocytes, microglia and oligodendroglia (Richter-Landsberg *et al.*, 2000; Mori *et al.*, 2002; Austin *et al.*, 2006). It is known to interact with a variety of proteins (Jenco *et al.*, 1998; Peng *et al.*, 2005) and lipid membranes (Jo *et al.*, 2000). The physiological functions of aSyn are still being established, but its interaction with pre-synaptic membranes and lipids suggests a role in the regulation of synaptic vesicle pools including dopamine release control (Perez and Hastings, 2004) and in lipid metabolism (Cabin *et al.*, 2002; Castagnet *et al.*, 2005; Golovko *et al.*, 2009).

Both in vitro and in vivo in LB, aSyn can self-assemble to form ordered fibrillar aggregates, characterized by a cross β-sheet structure, that are morphologically similar to the amyloid fibrils found in neuritic plaques in Alzheimer´s disease (AD) as well as in deposits associated with other amyloidogenic processes (Chiti and Dobson, 2006). The initial phase of the aggregation process is thought to involve the formation of intermediate oligomers and protofibrillar species which, according to accumulating experimental evidence, can be more toxic to cells than the mature fibrils into which they develop (Bucciantini *et al.*, 2002; Stefani and Dobson, 2003). These and other findings suggest a common structure-linked toxicity among pre-fibrillar species, and propose similar

mechanisms contributing to pathogenesis for this group of diseases (Bucciantini *et al.*, 2004; Baglioni *et al.*, 2006). Overall, different hypotheses have been proposed that postulate that aSyn induces a 'gain of toxic function' upon aggregation (Bennett, 2005).

While aSyn is typically considered as an intracellular protein, it has also been found to be normally present in extracellular biological fluids, including human cerebrospinal fluid (CSF) and blood plasma (Borghi *et al.*, 2000; El-Agnaf *et al.*, 2003, 2006; Lee *et al.*, 2006; Tokuda *et al.*, 2006). However, aSyn levels have been found to be elevated in plasma from PD and multiple system atrophy (MSA) patients relative to age-matched controls (Lee *et al.*, 2006), while lower levels than normal have been detected in CSF from PD patients (Tokuda *et al.*, 2006). On the other hand, two studies by El-Agnaf and colleagues showed an elevated content of oligomeric aSyn species present in plasma (El-Agnaf *et al.*, 2006) and post mortem CSF (Tokuda *et al.*, 2010) from PD patients, compared to controls, indicating that changes in the levels and characteristics of extracellular aSyn are associated with the disease (Lee, 2008). Even though membrane permeability from dying cells could be one contributing factor, it has been suggested that vesicle-mediated exocytosis from normal cells is probably the main source of extracellular aSyn (Lee, 2008). By using brain homogenates and neuronal cell cultures, Lee and colleagues (Lee, Patel and Lee, 2005) have shown that both monomeric and aggregated aSyn can be secreted by an unconventional secretory pathway. On the other hand, extracellular aSyn has been shown to be taken up by neuronal and microglial cells in culture, although the nature of the mechanism involved is still controversial (Lee, 2008). In addition, two recent studies have provided strong evidence for a neuron-to-neuron and neuron-to-non-neuronal cell transmission of aSyn aggregates and their associated cytotoxicity, in cellular and mouse

models of PD (Desplats *et al.*, 2009; Danzer *et al.*, 2011), highlighting the importance of extracellular aSyn in the pathogenic mechanism of α-synucleinopathies.

## 2. Neuroinflammation in PD

Another prominent pathological feature of PD brains is the presence of a robust inflammatory response mediated by activated microglia and reactive astrocytes in affected areas of the SN (Glass *et al.*, 2010). Inflammation is the first response of the immune system to pathogens or irritation. In acute conditions, it protects tissue against invading agents and promotes healing. However, a chronic inflammatory state can turn harmful towards the host´s own tissue (Kim and Joh, 2006; Gao and Hong, 2008). Microglia are the resident immunocompetent cells in the brain (Aloisi, 2001), capable of antigen presentation to lymphocytes (Kreutzberg, 1996) and rapid activation in response to immune insults and invading of PD pathogenesis in the central nervous system (CNS) (Kim and Joh, 2006). As a result of pathogen invasion or tissue damage, microglia switch to an activated phenotype and thereby promote an inflammatory response that serves to further engage the immune system by recruiting other cells to the site of brain lesion, and initiate tissue repair (Glass *et al.*, 2010). However, uncontrolled inflammation may result in production of neurotoxic factors that can be highly detrimental (Gao and Hong, 2008; Glass *et al.*, 2010). Indeed, inflammation in the CNS and sustained overactivation of microglia, i.e. reactive microgliosis, are currently believed to be actively involved in the pathogenesis of various neurodegenerative diseases including PD, AD, multiple sclerosis (MS), and amyotrophic lateral sclerosis (ALS) (Kim and Joh, 2006; Gao and Hong, 2008; Long-Smith, Sullivan and Nolan, 2009; Glass *et al.*, 2010).

At present, whether microglial activation ultimately protects or actually exacerbates neuronal loss in the context of PD and other related diseases is still under debate (Vila *et al.*, 2001; Wu *et al.*, 2002; Wyss-Coray and Mucke, 2002; Delgado and Ganea, 2003; Sánchez-Pernaute *et al.*, 2004; Gao and Hong, 2008; Halliday and Stevens, 2011), although the current view favours the second hypothesis. Evidence of microglial attack in PD is supported by findings from epidemiological studies, animal models, and cell culture experiments (McGeer and McGeer, 2008). Epidemiological studies have revealed that taking ibuprofen anti-inflammatory agent regularly is associated with a 35% lower risk of PD (Chen *et al.*, 2003, 2005), supporting the concept that inflammatory attack is contributing to dopaminergic neuronal loss. On the other hand, in vivo studies show that the specific early up-regulation of SN microglia in PD correlates with disease severity and dopamine terminal loss (Orr *et al.*, 2005; Ouchi *et al.*, 2005). Overall, studies based on animal models and in vitro cell culture, indicate that dopaminergic cells are highly sensitive to inflammatory attack (Castaño *et al.*, 1998; Fernagut and Chesselet, 2004) and that microglial cells can be strongly activated to mount such an inflammatory response (Austin *et al.*, 2006). Moreover, it has been recently reported that treatment with CSF from PD patients strongly affects cultured microglial cells, resulting in reduced cell growth, morphological changes, as well as increased content and aggregation of aSyn (Schiess *et al.*, 2010). This illustrates how microglia itself, and not only dopaminergic neurons, can be highly affected by the medium in a PD scenario.

## 3. αSyn-induced microglial activation

The results gathered thus far using the different PD animal models have substantially increased our understanding of PD's pathogenesis by usually providing different but probably complementary information. Thus, while the

MPTP mouse model of PD indicates that inflammation in the SN can be self-propagating and leads to progressive neurodegeneration, the aSyn transgenic animal model demonstrates that overexpression of this endogenous protein can certainly provide a powerful source of inflammation (McGeer and McGeer, 2008). Whether microglial activation is essentially caused by the release of aberrant aSyn species to the extracellular space, (Zhang *et al.*, 2005; Wersinger and Sidhu, 2006; Reynolds, Kadiu, *et al.*, 2008), or otherwise, that neuronal death itself drives microglial immune responses in an aSyn-independent manner (Giasson *et al.*, 2000; Przedborski *et al.*, 2001; Mandel *et al.*, 2005), is still under debate. However, there is ample accumulated evidence pointing at aSyn as the main trigger of microglial activation in PD (Roodveldt, Christodoulou and Dobson, 2008). For example, several studies have demonstrated that extracellular and nigral aSyn- containing aggregates are often surrounded by activated microglia or inflammatory mediators in PD brains (McGeer *et al.*, 1988; Yamada, McGeer and McGeer, 1992), similarly to what has been described for amyloid plaques in AD (Griffin *et al.*, 2006). Moreover, the extent of microglial activation in the SN from PD patients has been found to be correlated with the degree of aSyn accumulation (Croisier *et al.*, 2005) and with increased aSyn levels as evidenced by in vitro (Klegeris *et al.*, 2008; Kim *et al.*, 2009) and in vivo (Lee, Tran and Tansey, 2009) studies, strongly supporting the view that the protein has a major role in phenotypic changes of microglia. Up to this point, a considerable number of in vivo studies with animal models of PD that directly link aSyn with microglial activation have been reported. It has been demonstrated in mice that overexpression of aSyn alone (by using adeno-associated virus, AAV) is sufficient to trigger neuroinflammation, involving not only classical microglial activation but also stimulation of adaptive immunity, preceding the appearance of overt neurodegeneration signs (Theodore *et al.*, 2008). In line with this finding, a rat

AAV-based model for overexpressing the A53T aSyn variant in the SN revealed dramatic changes in cytoskeletal protein levels and activated microglia-mediated neuroinflammation in the striatum (with increased release levels of IL-1β, IFN-γ, and TNF-α proinflammatory cytokines), well before neuronal loss was evident (Chung *et al.*, 2009). Another recent study using a AAV rat PD model showed that overexpression of Wt aSyn in the SN not only leads to persistent microglia activation, but that depending on the degree of aSyn-induced neurophatology that models either the onset or the late stages of the disease, different microglial responses will occur: upon lower aSyn expression levels where only neurodegeneration occurs, microglia with antigen-presenting capabilities predominate, whereas levels that can induce neuronal cell death correlate with long-term induction of macrophagic microglia (Sanchez-Guajardo *et al.*, 2010), suggesting that microglia may play different roles during disease progression (Sanchez-Guajardo *et al.*, 2010).

Two recent studies have explored the link between neuroinflammation and aSyn dysfunction by lipopolysacharide (LPS) injection in rat (Choi, Zhang and Bing, 2010) or mice (Gao *et al.*, 2011), to trigger systemic and brain inflammation. In the first study, the authors observed increased microglia activation and secretion of proinflammatory cytokines as well as greater nitration of proteins including aSyn, in elderly rats, suggesting that an exaggerated neuroinflammatory response that occurs naturally with aging might contribute to aSyn aggregation and dopaminergic neurodegeneration in PD (Choi, Zhang and Bing, 2010). In the second study, the authors evaluated dopaminergic neurodegeneration, aSyn pathology and neuroinflammation in Wt and transgenic A53T aSyn-overexpressing mice (Gao *et al.*, 2011). They observed that, while both models initially displayed acute neuroinflammation, only the latter developed persistent neuroinflammation together with chronic

progressive degeneration of nigrostriatal dopamine pathway, accumulation of aggregated, nitrated aSyn, and formation of LB (Gao *et al.*, 2011), suggesting that genetic factors and environmental exposures act synergistically to precipitate the development of PD. On the other hand, microglial cells from aSyn-knockout mice have been shown to exhibit a remarkably different morphology compared to Wt cells (Austin *et al.*, 2006), displaying elevated levels of secreted pro-inflammatory cytokines such as TNF-α and IL-6 after activation, indicating that aSyn plays a critical role in modulating the microglial activation state. More recently, the authors have found that microglial activation in this model is accompanied by increased protein levels of three enzymes involved in lipid-mediated signalling, which suggests a broader function for aSyn in brain physiology beyond synapsis control (Austin *et al.*, 2011).

In the last few years, several in vitro studies have focused on the effects of extracellular aSyn on microglial activation. Zhang et al. (Zhang *et al.*, 2005) first reported that exogenous, aggregated aSyn cause activation of microglial cells, which then become toxic towards cultured dopaminergic neurons. Their results indicate that microglial phagocytosis of aSyn and activation of NADPH oxidase, are critical in aSyn-induced microglial activation and neurotoxicity. This finding is highly relevant considering that aggregated aSyn has been shown to be secreted by exocytosis from neuroblastoma and primary neuronal cells (Lee, Patel and Lee, 2005; Danzer *et al.*, 2011), and by stressed neurons (Klegeris *et al.*, 2008). Moreover, following the discovery that aSyn aggregates can be released from neurons and transmitted to neighbouring cells (Desplats *et al.*, 2009), a study has recently shown that aSyn release by SH-SY5Y neuroglioma cells, especially when treated with MPP+ neurotoxin, are able to activate the inflammatory response in a microglial cell line (Alvarez-Erviti *et al.*, 2011).

Up to this point, research on aSyn-mediated cell response has focused primarily on the effects on neuroinflammation (Benner *et al.*, 2008) or microglial activation (Zhang *et al.*, 2005, 2007; Thomas *et al.*, 2007; Reynolds, Glanzer, *et al.*, 2008; Cookson, 2009) of aSyn in its aggregated form. Interestingly, Reynolds and co-workers (Reynolds, Kadiu, *et al.*, 2008) have found that nitrated, aggregated aSyn (N-aSyn) has a stronger stimulating effect on microglia than that of nitrated but non-aggregated aSyn. In addition, several investigations have found that N-aSyn, which has been detected in LB of human brains with PD (Giasson *et al.*, 2000) and has been linked to neurodegeneration in PD mouse models (Benner *et al.*, 2008; Gao *et al.*, 2008), induces a neurotoxic inflammatory microglial phenotype that accelerates dopaminergic neuronal loss (Biasini *et al.*, 2004; Zhang *et al.*, 2005; Zhou *et al.*, 2005; Thomas *et al.*, 2007). By integrating genomic and proteomic techniques, Gendelman and colleagues created a fingerprint of microglial cell activation following its interactions with aggregated N-aSyn in cell culture (Reynolds, Kadiu, *et al.*, 2008), indicating that the activation, which was found to be capable of mediating dopaminergic neurotoxicity, is mainly mediated by the NF-κB pathway (Reynolds, Kadiu, *et al.*, 2008). However, whether extracellular aSyn contains the same modifications than the protein found in LB (Giasson *et al.*, 2000; Hodara *et al.*, 2004; Anderson *et al.*, 2006), which is a typically pro-oxidative environment, is still uncertain (Lee, 2008).

Over the last few years, certain differential functions for non-aggregated, extracellular aSyn in glia have been reported. It has been observed that, in contrast to the aggregated form, monomeric aSyn enhances microglial phagocytosis (Park *et al.*, 2008). A few investigations that explore the effects of non-aggregated aSyn on the cytokine release profile of potentially relevant cells have been recently done using monocytic (Klegeris *et al.*, 2008) or macrophage

(Lee *et al.*, 2009) cell lines, and primary astrocyte (Klegeris *et al.*, 2006) or microglial (Su *et al.*, 2008; Su, Federoff and Maguire-Zeiss, 2009; Roodveldt *et al.*, 2010) cultures. Indeed, we have observed a strong innate immune response in primary glial and microglial cell cultures elicited by exogenous, non-aggregated aSyn (Roodveldt *et al.*, 2010). Interestingly, a comparative study using unmodified aSyn has recently shown that exogenous non-aggregated aSyn induces higher TNF-α, IL-1β and ROS release levels than aggregated aSyn in microglia (Lee *et al.*, 2010). These and other recent findings point at the importance of exploring the effects on the immune response of aggregated as well as non-aggregated aSyn.

Even though a study using monocytic THP-1 cell line (Klegeris *et al.*, 2008) had shown modest increases in IL-1β and TNF-α secretion levels after stimulation with A30P, A53T, or E46K aSyn mutants compared to the Wt protein, there is a lack of a comprehensive study of the effect exerted by non-aggregated aSyn, performed with primary cell cultures. With this in mind, we analysed the cytokine release profile of primary microglial cultures —which represents a more comparable physiological environment— after stimulation with Wt or the PD-linked aSyn mutants (Roodveldt *et al.*, 2010). Indeed, we found remarkable differences between the aSyn variants in the interleukin and chemokine release profiles and significant effects on the microglial phagocytic capacity (Roodveldt *et al.*, 2010). In particular, we observed marked differences in IL-6 and IL-1β pro-inflammtory cytokines, IL-10 immunoregulatory cytokine, as well as IP-10/CXCL10, RANTES/CCL5, MCP-1/CCL2 and MIP-1α/CCL3 chemokines release levels. Our results indicate that extracellular, non-aggregated Wt aSyn produces a moderate to low pro-inflammatory response in glia, together with a reduction of the immunoregulatory response, and a moderate stimulation of Th1 chemokine secretion. The A30P and E46K pathological variants, on the other

hand, can induce strong pro-inflammatory and immunoregulatory responses, together with marked increases in chemokine release levels. This exacerbated innate immune response might explain the earlier onset and more rapid evolution of these two genetic cases of PD as compared to the sporadic kind. Intriguingly, our results from the pathologically-linked A53T variant showed not to provoke a significant innate immune response, which might suggest that other neurodegeneration mechanisms contributing to the pathogenesis of PD, probably involving the adaptive immune response, might exist in this case. Combined with the effect on microglial phagocytosis, our results indicate that these aSyn-induced phenotypes might reflect either a classical (A30P and E36K) or an alternative (A53T) microglial activation state, or a hybrid phenotype (Wt), which could probably explain the different disease progression modes that can occur in PD. Alternative activation of macrophages and microglia is a response to tissue injury that is thought to be involved in tissue repair and restoration (Ponomarev *et al.*, 2007), and has been suggested to play a role in repair and extracellular matrix remodelling in AD (Colton *et al.*, 2006). Currently, there is no other indication that such an activation mode could be operating in the context of PD.

Upon activation, microglia and astrocytes start secreting inflammatory cytokines in order to communicate and mount the immune response to counteract disease or injury. The cytokines TNF-α, IL-1β, IL-2, IL-4, IL-6, TGF-α, TGF-β1, TGF-β2 have all been reported to be increased in the nigrostriatal region and CSF of patients with PD or DLB (Croisier and Graeber, 2006). As a result of aSyn-induced activation of microglia in vitro, a few cytokines and metabolites have been shown to be significantly up-regulated (reviewed in (Roodveldt et al., 2008)): IL-6, IL-1β, ICAM-1, TNF-α, IFN-γ, MCP-1, O2-, iROS, and PEG2, glutamate, and iCys. In general, disease-linked aSyn variants show a

stronger effect on cytokines release than does the Wt protein. Interestingly, analysis of the microglia transcriptome by Gendelman and co-workers (Reynolds, Glanzer, *et al.*, 2008) after stimulation with aggregated N-aSyn, revealed a significant up-regulation of the toll-like receptor 2 (TLR-2) gene. TLRs are known to sense the molecular signatures of microbial pathogens, and play a fundamental role in innate immune responses, inducing the expression of diverse inflammatory genes (Kawai and Akira, 2007). Therefore, it seems plausible that cells challenged with aSyn, or with certain forms of aSyn, could become hyper-responsive to inflammatory signals.

Activated microglia can also produce substantial amounts of superoxide radicals, which may be the major source of the oxidative stress thought to be largely responsible for dopaminergic cell death in PD. The generation of ROS by microglia activated by aSyn (Thomas *et al.*, 2007) can result in oxidation and nitration of proteins, DNA modifications, and lipid peroxidation, leading to neurotoxicity (Zhang *et al.*, 2005). Oxidation (Ko *et al.*, 2000; Souza *et al.*, 2000) and nitration (Giasson *et al.*, 2000; Souza *et al.*, 2000) of aSyn, in turn, can lead to the formation of more aggregates, which could result in increased cytotoxic effects. Consistent with this, Kelly et al. have shown that high levels of oxidized cholesterol metabolites in brains from PD and dementia with LB patients, accelerate aSyn fibrillation (Bosco *et al.*, 2006). On the other hand, McGeer and colleagues (Klegeris *et al.*, 2007) have reported that aSyn-stimulated microglia, in combination with IFN-γ, produce and increase in the toxicity on human monocytic cells exerted by neurotoxic secretions (Klegeris *et al.*, 2007). Interestingly, this toxicity can be diminished with specific ligands for ryanodine receptors (Klegeris *et al.*, 2007), which are known to help mediate the efflux of Ca2+ ions from intracellular stores and avoid uncontrolled increases in $[Ca2+]_i$ that may lead to cell death (Giorgi *et al.*, 2008).

Further insight into the mechanism of pathogenesis might derive from the findings that several proteins which are thought to be linked to PD are up-regulated as a result of aSyn-induced microglial activation. Gendelman and co-workers, by determining the activated microglia proteome profile (Reynolds, Glanzer, *et al.*, 2008), found that aggregated N-aSyn activation of microglia results in differential expression of several proteins. These range from proteins involved in oxidative stress, cell adhesion, glycolysis, growth control, and migration, to cytoskeletal proteins. It is worth noting that two of those proteins found to be most up-regulated, calmodulin and ubiquitin, have been shown to interact with aSyn with possible functional consequences. Calmodulin has been shown, in vitro, to bind to aSyn in a Ca2+-dependent manner (Lee *et al.*, 2002) and to inhibit aSyn fibrillation (Martinez *et al.*, 2003). On the other hand, a fraction of aSyn found in LB is mono-ubiquitinated (Hasegawa *et al.*, 2002; Tofaris *et al.*, 2003), but the role of this modification remains unclear. Recently, it has been demonstrated that the ubiquitin-protein isopeptide ligase, seven in absentia homolog (SIAH), directly interacts with and monoubiquitinates aSyn, promoting its aggregation (Lee *et al.*, 2008; Rott *et al.*, 2008) and apoptosis (Lee *et al.*, 2008). In addition, there is also evidence implicating a role for the ubiquitin-proteasome system (UPS) in PD (reviewed in (Lim and Tan, 2007). Also of interest are the elevated levels of Hsp70 observed upon microglial activation. This central chaperone has been demonstrated to inhibit aSyn aggregation in vitro (Dedmon *et al.*, 2005; Huang *et al.*, 2006; Roodveldt *et al.*, 2009), in neuroglioma cells (Klucken *et al.*, 2004) as well as in fly (Auluck *et al.*, 2002) and mouse (Klucken *et al.*, 2004) models of PD, protecting cells from the cytotoxic effects of aggregates.

## 4. Links between aSyn and astrocytes and oligodendrocytes

Together with microglial cells, astrocytes and oligodendrocytes are part of glia, which normally serve neuroprotective functions but, given adverse stimulation as discussed before, they may contribute to develop chronic neuroinflammation (McGeer and McGeer, 2008; Halliday and Stevens, 2011). Compared to microglia, the functions of astrocytes are poorly understood. Because they have been shown to produce both pro-inflammatory and anti-inflammatory agents, these cells are thought to have a dual role (McGeer and McGeer, 2008). Many ICAM-1-positive astrocytes are seen in the SN of PD brains and this may attract reactive microglia to the area since microglia carry the counter receptor LFA-1 (Miklossy *et al.*, 2006). Indeed, aSyn has been shown to be capable of both of activating microglia and stimulating astrocytes to produce IL-6 and ICAM-1 (Klegeris *et al.*, 2006). On the other hand, astrocytes have been shown to secrete a number of neurotrophic factors that protect dopaminergic neurons in some models of PD (McGeer and McGeer, 2008), but the mechanisms underlying most of these functions are not yet known. Astrocytes have been shown to express aSyn (Tanji *et al.*, 2001). Interestingly, the presence of aSyn-containing inclusion bodies in astrocytes of sporadic PD brains has been observed (Wakabayashi *et al.*, 2000; Terada *et al.*, 2003; Braak, Sastre and Del Tredici, 2007). Finally, a recent study showed that astrocyte expression of A53T aSyn leads to the development of progressed paralysis, strong microglial activation, and neurodegeneration (Gu *et al.*, 2010).

There is still little data on the role of oligodendrocytes in PD. aSyn-containing inclusions have been detected in this cell type in MSA, in DLB, and in PD (Wakabayashi *et al.*, 2000; Campbell *et al.*, 2001). McGeer and colleagues have reported the presence of complement-activated oligodendrocytes in the SN of PD cases (Yamada, McGeer and McGeer, 1992). Intriguingly, transgenic mice overexpressing Wt aSyn in oligodendrocytes have been observed to develop

severe neurological alterations and neurodegeneration (Shults *et al.*, 2005; Yazawa *et al.*, 2005), drawing the attention to a possible role of these glial cells in PD.

## 5. Expression of aSyn by immunocompetent cells

Given that aSyn expression has been reported also in non-neuronal cells, it is currently thought to play a role besides dopamine release control. While searching for a link between the CNS and peripheral immune system in PD, Kim et al. (Kim *et al.*, 2004) found that aSyn was up-regulated in peripheral blood mononuclear cells (PBMC) at the gene level, in idiopathic PD vs. non-PD controls. Moreover, by in vitro transfection with Wt, A30P and A53T aSyn genes, they found that aSyn expression is correlated to glucocorticoid-sensitive apoptosis, possibly caused by the enhanced expression of glucocorticoid receptor (GR), caspase activation, CD95 (Fas) up-regulation, and ROS production. However, the increase in ROS production by overexpression of the aSyn mutants was markedly greater than for Wt aSyn. aSyn expression has also been found in cultured human macrophages (Tanji *et al.*, 2002) and its expression levels have been reported to increase by stimulation with proinflammatory cytokine IL-1β or LPS (Tanji et al., 2002), further supporting a role for aSyn in the inflammatory process. Finally, expression of aSyn in cultured human T cells, B cells, natural killer (NK) cells and in monocytes/macrophages, have been reported (Shin *et al.*, 2000). Currently, it is not known whether expression or aggregation, of aSyn in T cells can be regulated by ligand activation of the T cell. This may be relevant as it could represent a key link between regulation of the adaptive immunity and aSyn expression levels.

## 6. aSyn and the adaptive immune response in PD

In the last few years, mounting evidence has pointed at a possible participation of the adaptive immune system in PD pathogenesis. However, whether this immune response actually contributes to neurodegeneration, and in that case by which mechanism, remains unknown. The initial observations in PD patients that a small amount of CD8+ T lymphocytes occur in proximity to degenerating nigral neurons (McGeer *et al.*, 1988), and the occurrence in LB of components of the classical or antibody-triggered complement cascade (Yamada, McGeer and McGeer, 1992) had suggested a possible involvement of the adaptive immunity in the PD process. More recently, the finding of accumulated IgG in the SN of PD patients and increased expression of IgG-binding receptors on activated microglia (Orr *et al.*, 2005), and the detection of anti-aSyn autoantibodies (AAb) in blood serum of PD patients (Papachroni *et al.*, 2007), suggest that the pathological process may involve adaptive immune-mediated mechanisms. In addition, the observation that humoral immune mechanisms can trigger microglial-mediated neuronal injury in animal models of PD (He, Le and Appel, 2002), and the finding by Standaert and colleagues of IgG deposition in mouse brains following AAV-mediated aSyn overexpression in the SN (Theodore *et al.*, 2008), further support a role of the adaptive immune system in disease progression.

A possible consequence of the initial microglial activation in the affected regions of PD brains is the local permeabilization of the blood-brain barrier (BBB), leading to infiltration to the affected regions by B- and/or T-lymphocytes (Racke *et al.*, 2000). Indeed, a remarkable T- and B-cell infiltration into the SN linked to aSyn overexpression was observed at the early stages, i.e. before the appearance of significant dopaminergic neuronal loss, reaching levels in the SN of up to 10-fold and 5-fold compared to controls (Theodore *et al.*, 2008). A recent study by Hunot and colleagues (Brochard *et al.*, 2009) has shown that

CD8+ and CD4+ T-cells, but not B-cells, had invaded the brain in PD patients and in MPTP-treated mice during the course of neural degeneration. Furthermore, based on these results the authors propose that T-cell dependant toxicity is essentially mediated by CD4+ T-cells and requires the expression of FasL (Brochard *et al.*, 2009). Given that the FasL pathway had been shown to produce proinflammatory cytokine responses in macrophages (Park *et al.*, 2003), the authors speculate that the CD4+ Th FasL-mediated activation of microglia could participate in neuroinflammation and neurodegeneration processes in PD (Brochard *et al.*, 2009).

Based on results obtained with an MPTP murine model of the disease, Gendelman and colleagues (Reynolds *et al.*, 2010) have suggested that the aSyn-specific regulatory T-cells (Treg cells), which are regulatory components of the adaptive immunity, might be able to counteract the autoaggresive effector T-cell responses that exacerbate neuroinflammation (Benner *et al.*, 2008), and therefore contribute to attenuate neurodegeneration in PD. Indeed, the same group has reported that microglial cells stimulated with N-aSyn are susceptible of essentially opposing immune regulatory responses by Treg cells (CD4+, CD245+) and effector T-cells (CD4+, CD25-) in culture (A. D. Reynolds *et al.*, 2009). By analysing an array of cytokines released by treated microglia, the authors found that, while the effector T-cell subset exacerbates microglial-mediated inflammation and may induce neurotoxic responses, Treg cells are able to suppress N-aSyn microglial-induced reactive-oxygen species (ROS) and NF-κB activation and are proposed to be neuroprotective (A. D. Reynolds *et al.*, 2009). Furthermore, the study indicates that Treg cells can regulate microglial inflammation by inducing Fas-FasL-mediated apoptosis of N-aSyn-stimulated microglial cells (A. D. Reynolds *et al.*, 2009). By using a proteomic analysis, the authors further showed that these Treg cells can significantly alter the

microglial protein expression profile for certain proteins linked to cell metabolism, migration, protein transport and degradation, redox biology, and cytoskeletal and bioenergetics metabolism, to presumably attenuate the neurotoxic phenotype caused by N-aSyn stimulation (Ashley D. Reynolds *et al.*, 2009).

Thus far, accumulated data demonstrate that in the MPTP model of PD, misfolded and aggregated aSyn are secreted from neurons, which promotes pro-inflammatory M1-type microglia and cytotoxic T-cells, therefore amplifying neuronal damage. In sporadic human PD, it is currently unkown which factor triggers disease onset, but it has been proposed that under certain circumstances, a similar set of temporal and mechanistic events could transform neuroprotective microglia and T cells into cytotoxic cells, thereby accelerating disease progression (Appel, Beers and Henkel, 2010). This way, activated microglia and the cytokine milieu that they generate might promote T-cell differentiation into different cell subsets in the context of PD (Appel, Beers and Henkel, 2010). Indeed, it has been shown that M1 (pro-inflammatory) cells promote, whereas M2 (non-inflammatory) cells reduce, CD4+ Th1 cell proliferation and function (Verreck *et al.*, 2004), but also that, conversely, T-cells can dictate microglial pro- or anti-inflammatory phenotypes (Giuliani *et al.*, 2003; Kebir *et al.*, 2007; Mount *et al.*, 2007). Whether microglia dictate the specific T-cell phenotype or otherwise, that T-cells dictate the specific microglial phenotype (i.e. M1 vs. M2), is still unknown (Appel, Beers and Henkel, 2010). But overall, the communication established between microglia, T cells and neurons seem to indicate that the immune response is not only a consequence of injury, but that it actively contributes to the balance between neuroprotection and neurotoxicity (Stone *et al.*, 2009; Appel, Beers and Henkel, 2010).

To analyse the possibility that humoral immunity may play a role in initiating or regulating inflammation, Orr et al. (Orr *et al.*, 2005) analysed the association between nigral degeneration and humoral immune markers in brain tissue of patients with idiopathic or genetic PD and controls. All the patients with PD revealed IgG, but not IgM, binding on dopamine neurons. Moreover, the proportion of IgG-immunopositive neurons showed a negative correlation with the degree of cell loss in the SN, and positive correlation with the number of activated microglia. IgG was found to be concentrated at the cell surface of neurons, but also on their LB, and was shown to co-localize with aSyn. These results, in combination with their finding that activated microglia express high-affinity IgG receptors (FcγRI) in both idiopathic and genetic forms of PD, might suggest that the activation of microglia may be induced by neuronal IgG (Orr *et al.*, 2005).

The question regarding the functional importance of antibodies against antigen-specific, disease-associated neuronal proteins still needs to be addressed. It has been demonstrated that an IgG fraction purified from serum of PD patients causes death of dopaminergic neurons in vivo following stereotaxic injection in the SN of experimental animals (Chen *et al.*, 1998), and the presence of immunoglobulins in PD brain tissue have been proposed to lead to the targeting of dopaminergic nigral neurons for destruction (Orr *et al.*, 2005). Currently, it remains unknown whether these anti-aSyn AAb are neurotoxic, or on the contrary, they actually have a neuroprotective role, as has been shown in a human aSyn transgenic mouse model of PD (Masliah *et al.*, 2005).

A recent study has assessed the presence of auto-antibodies (AAb) against all three synucleins in the peripheral blood serum of PD patients and healthy controls (Papachroni *et al.*, 2007). While the presence of AAb against β- and γ-

Syn showed no association with PD, multi-epitopic AAb against aSyn were detected in 65% of all patients, with a strong correlation with the inherited mode of the disease. In addition, a recent study based on measuring AAb levels against monomeric, oligomeric, and fibrillar aSyn in serum from PD patients (Gruden *et al.*, 2011), showed that all three AAb specificities reached the highest values after 5-year of disease duration, and subsided in 10-year sufferers. Intriguingly, there was a ca. 15-fold increase in AAb titre values relative to monomeric aSyn (72% of patients), and a ca. 4-fold increase for aSyn oligomers (56% of patients). Moreover, the authors also found a decline in CD3+, CD4+ and CD8+ T-lymphocyte and B-lymphocyte subsets. Based on these results, they suggest that aSyn toxicity elimination by AAb in early PD pathology might be linked with the decline of lymphocyte subsets reflecting the influence of inflammatory and oxidative stress processes (Gruden *et al.*, 2011).

Despite their potential involvement in PD pathogenesis and progression, the role of NK cells in PD has hardly been explored. NK cells are active members of the innate immune system that act as a first-line defence, and also mediate between the innate and adaptive immune systems (Salazar-Mather, Ishikawa and Biron, 1996; Su *et al.*, 2001). Interestingly, a recent study using blood samples from PD patiens indicates that the NK activity increases as the disease advances (Mihara *et al.*, 2008). Moreover, the study also showed that the NK cell content among the total lymphocytes of the PD group was higher than in the control group (Mihara *et al.*, 2008).

## 7. Prospects for aSyn-based immunotherapy in PD

In addition to its well-known importance in the pathogenesis of PD, aSyn is becoming a primary target for preventing or controlling the process of PD. In the late few years, vaccination for treating some neurodegenerative disorders

has emerged as a potentially useful approach. Thus far, this avenue has been scarcely explored for PD. Importantly, immunization with aSyn was shown to generate a humoral response in a mouse model of PD (Masliah *et al.*, 2005), producing beneficial albeit modest results on histopathological markers of the disease. On the contrary, using N-aSyn as the immunogen proved to elicit strong antigen-specific effector T cell responses in MPTP-intoxicated mice that caused exacerbated neuroinflammation and neurodegeneration (Benner *et al.*, 2008). This response was further shown to be largely mediated by Th17 cells and causing Treg dysfunction (Reynolds *et al.*, 2010). In addition, the authors demonstrated that Treg cells from mice treated with the neuropeptide VIP, known to promote Treg responses (Delgado *et al.*, 2005; Gonzalez-Rey *et al.*, 2006), can efficiently modulate N-aSyn-generated immunity in MPTP mice and confer neuroprotection (Reynolds *et al.*, 2010), suggesting a possible novel therapeutic avenue for PD.

Given that microglial activation can maintain or even aggravate the disease process, blocking inflammation or shifting the balance between pro-inflammatory and anti-inflammatory states in a controlled manner, offers one of the most promising strategies for developing palliative (and maybe preventive) therapies for PD and related disorders. Epidemiological data have identified the non-steroidal anti-inflammatory drug (NSAID) ibuprofen as neuroprotective for PD (Klegeris, McGeer and McGeer, 2007). NSAIDs are thought to act on dopamine quinone formation and activation by aSyn of both astrocytes and microglia. On the other hand, Gendelman and colleagues demonstrated that T cells from mice immunized with Copolymer-1 (Cop-1), are able to attenuate microglial responses and lead to neuroprotection in a MPTP mouse model of PD (Benner *et al.*, 2004). This neuroprotective effect was later found to be mediated by the CD4+ type of T cells, suggesting the possible

involvement of Treg cells (Laurie *et al.*, 2007). Later work by the same group confirmed this hypothesis by showing that passive transfer to MPTP mice of activated Treg cells, but not effector T cells, efficiently suppressed microglial activation and afforded neuroprotection (Reynolds *et al.*, 2007), suggesting that the immunomodulating abilities of Treg cells could potentially be utilized as a therapeutic approach against PD (Stone *et al.*, 2009).

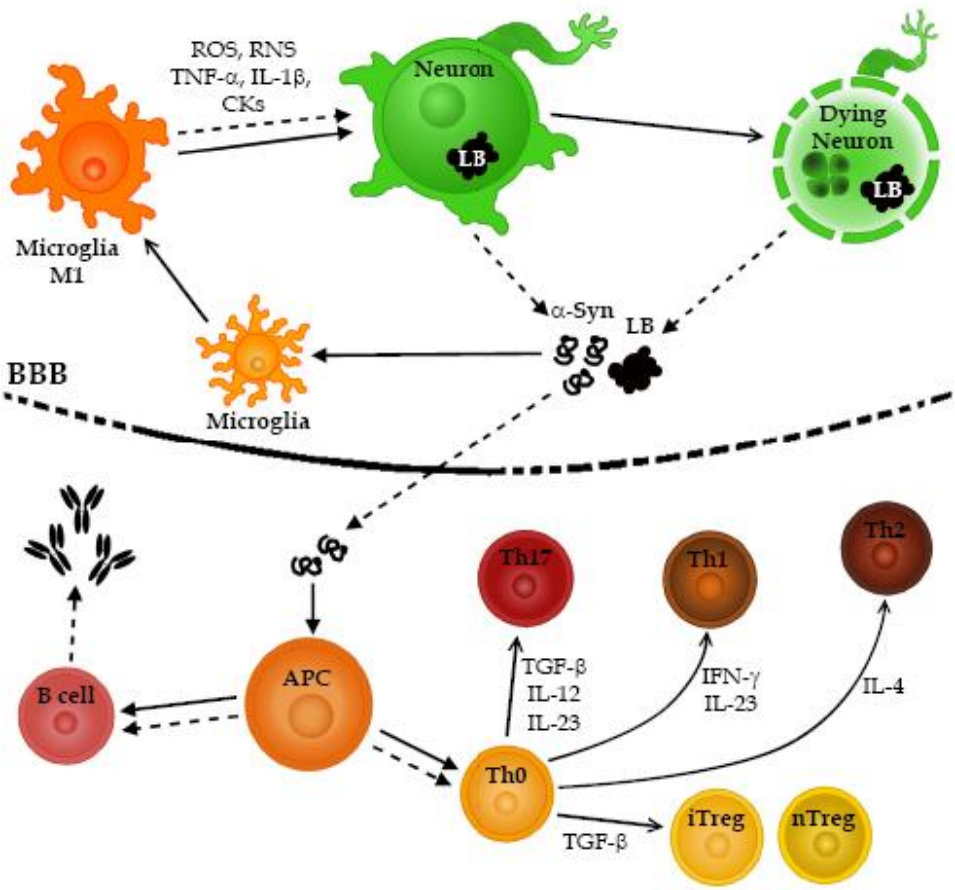


**Fig. 1. Links between aSyn and the immune response in PD**. Continuous arrows with filled tips: positive effect; continuous arrows with open tips: cell transformation; discontinuous arrows: secretion of cellular factors. APC, Antigen-processing cell; BBB, Blood-brain barrier; CKs, Chemokines; IL, Interleukin; IFN-γ, Interferon γ; LB, Lewy bodies; ROS, Reactive oxygen species; RNS, Reactive nitrogen species; TGF-β, Tumor growth factor β.

## 8. Conclusion

It is well established that PD onset and progression are characterized by sustained activation of microglia, linked to significant dopaminergic neuron loss in the SN of the brain. Over the last protein, is linked to neurotoxicity through various proposed mechanisms, and may be one of the primary causes of the immunological abnormalities observed in PD. Recent studies with cellular and in vivo models of the disease indicate that increased levels of extracellular aSyn, both in its aggregated and non-aggregated forms, are found in a PD scenario.

Accumulated evidence has now established that aggregated extracellular aSyn is able to trigger the activation of microglia, inducing a highly detrimental cascade of neuroinflammation and neuronal demise. In addition, recent studies have have demonstrated that non-aggregated aSyn can also have a substantial impact on microglial phenotype and cytokine release profile, especially in the cases of familial PD aSyn mutants. By releasing toxic factors, or by phagocytosing neighbouring cells, activated microglia and astrocytes are believed to form a self-perpetuating neuronal degeneration cycle. On the other hand, recent findings point at a possible role of the adaptive immune system involving aSyn, and the pathological process in PD. Clearly, further studies in this direction are necessary to help understand the complex immunological mechanisms underlying PD and the key, and possibly multiple, links between aSyn and the immune response in relation to in relation to pathogenesis (**Figure 1**).

In addition to trying to develop effective tools to prevent aSyn aggregation, modulating the innate immune response by intervening microglial activation, promoting a selective aSyn-specific humoral response, and manipulating the balance between effector and immunomodulatory T-cell populations, may be

considered as highly promising therapeutic approaches for the treatment of PD and other synucleinopathies.

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# The Hsp70 Chaperone System in Parkinson's Disease

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## 1. Introduction

Several neurodegenerative diseases are associated with a build up of misfolded or abnormal proteins and the formation of distinct aggregates, resulting in a putative pathological protein load on the nervous system (Chiti & Dobson 2006). This aberrant accumulation of amyloid or amyloid-like aggregates occurs in Parkinson's (PD), Alzheimer's (AD), and Huntington's (HD) diseases, amyotrophic lateral sclerosis, and frontotemporal dementia, among others. A broad array of cellular defence mechanisms operate to counteract this effect, including antioxidant proteins, the stress-inducible response and, in particular, molecular chaperones (Morimoto 2008; Voisine et al. 2010). Molecular chaperones are responsible for maintaining normal protein homeostasis within the cell by assisting protein folding, inhibiting protein aggregation, and modulating protein degradation pathways (Hartl & Hayer-Hartl 2009). Currently, there is substantial evidence supporting the involvement of these protein aggregational processes and a role of molecular chaperones, and especially of Hsp70, in PD pathogenesis (Bandopadhyay & de Belleroche 2010; Broadley & Hartl 2009; Witt 2010). Firstly, extensive colocalization of Hsp70 with α- synuclein (aSyn), the major component of Lewy bodies (LBs) (Spillantini et al. 1998), within the intraneuronal inclusions in PD brains has been

demonstrated (Auluck et al. 2002; McLean et al. 2002). Secondly, patients with PD show highly perturbed expression of different members of the Hsp70 family in the substantia nigra pars compacta (SN) of the brain, which is precisely the target of neurodegeneration (Grünblatt et al. 2001; Hauser et al. 2005). Finally, there is a considerable amount of data derived from studies performed in vitro, in cell culture and with animal models of PD (Arawaka et al. 2010; Witt 2010), that support the protective effects of Hsp70 against aSyn aggregation and toxicity, considered to be central in the aetiology of the disease.

The discovery within the last few years of three different missense mutations (A30P, E46K and A53T) in the aSyn gene as causative of early onset PD unambiguously linked this protein to disease onset and progression (Krüger et al. 1998; Polymeropoulos et al. 1997; Zarranz et al. 2004). Additionally, a locus triplication causing an increased dosage of the wild-type (Wt) aSyn gene has been found to potentiate neurodegeneration (Singleton et al. 2003). Finally, as mentioned above, aSyn is the major component of intracellular protein rich aggregates found in the brain of post-mortem patients of PD, the LBs and Lewy neurites (LNs). The appealing hypothesis for LBs formation is that aSyn monomers combine to form oligomers (or protofibrils), which coalesce into fibrils and then co-aggregate with other proteins into (intracellular) inclusions (Conway et al. 1998; Wood et al. 1999). While the monomers and oligomers of aSyn are soluble, the fibrils and LBs are insoluble in the neuronal cytoplasm. Some controversy arises, however, from the roles of the various physical forms or species of aSyn in PD pathogenesis. LBs have been proposed to be both neurotoxic (El-Agnaf et al. 1998), and protective (Mouradian 2002; Rochet et al. 2000). Other hypotheses state that the pre-fibrillar intermediates, composed of aSyn oligomers, are the main toxic species towards dopaminergic neurons (Conway et al. 2000; Volles & Lansbury 2003). Lansbury and co-workers have

shown that aSyn oligomers can form annular, elliptical, or circular amyloid pores in cell membranes (Lashuel, Hartley, et al. 2002; Volles & Lansbury 2003), and cell culture studies have demonstrated that aSyn oligomers reduce cell viability, disrupt lysosomes and induce Golgi fragmentation (Gosavi et al. 2002), as well as toxicity in animal models (Karpinar et al. 2009). In line with these findings, the more neurotoxic A30P and A53T mutants of aSyn share an increased tendency to form soluble oligomeric intermediates, whereas the E46K and A53T mutants fibrillate faster than the wild-type protein (Conway et al. 2000; Choi et al. 2004).

The heat-shock-protein 70 (Hsp70) family of chaperones (Mayer & Bukau 2005; Young 2010) is well conserved from bacteria to higher eukaryotes (where it is found within different organelles), having critical roles in a range of cellular processes such as promoting the folding of newly synthesized proteins and assisting the rescue of misfolded aggregated proteins. Hsp70 is highly relevant in the context of protein conformational diseases given that stress-induced cytosolic Hsp70 can prevent protein aggregation and enables the cell to avoid the accumulation of potentially toxic aggregates (Hartl 1996).

The structures of several Hsp70 homologues are similar and consist of an actin-like ATPase domain (nucleotide-binding domain, NBD) and a C-terminal substrate-binding domain (SBD), which are connected by a short linker region (Mayer & Bukau 2005). The substrate binding pocket recognizes and binds to unstructured or partially folded stretches within polypeptides (Bukau & Horwich 1998), with the current view that Hsp70s could prevent misfolding by binding to certain patterns in the polypeptide chain of the substrate that are highly enriched in hydrophobic residues (Maeda et al. 2007; Rüdiger et al. 1997). Even though most of our current understanding of the Hsp70 molecular mechanism has largely derived from studies performed with the bacterial

orthologue (DnaK), the outlines of the mechanism appear conserved (Hartl & Hayer-Hartl 2002; Young 2010). The ATPase cycle of Hsp70 involves alternation between an ATP-bound state which has low affinity and fast exchange rates for peptide substrates ('open' state), and an ADP-bound state with high affinity and low exchange rates for substrates ('closed' state) (Mayer & Bukau 2005). This alternation is achieved by a bidirectional structural communication between the NBD and the SBD domains, driven by a complex allosteric mechanism (Mayer & Bukau 2005; Young 2010).

The ATPase cycle is typically modulated by several co-chaperones, most notably the 'Jdomain' protein Hsp40/DNAJB1 (or DnaJ, the bacterial orthologue), resulting in increase of the ATPase activity (Bukau & Horwich 1998; Mayer & Bukau 2005; Minami et al. 1996), and the BAG family of proteins which function as nucleotide-exchange factors (NEFs) and promote the ADP release from the the Hsp70 NBD (Takayama et al. 1999; Young 2010). In addition to acting as enhancer of the basal ATPase rate of Hsp70, the Hsp40 family of co-chaperones has a key role in the canonical model of the Hsp70 machinery mechanism, given the ability of some of them to also recognize and bind to unfolded client proteins and 'deliver' them to Hsp70 (Kampinga & Craig 2010). Other important co-chaperones are Hop, which binds to the C-termini of Hsp70 and Hsp90 and assists substrate transfer between the two chaperones (Scheufler et al. 2000), and Hip (ST13) which has been shown to specifically bind to and stabilize, the ADP-bound state of Hsp70 (Höhfeld et al. 1995; Prapapanich et al. 1996), and has been suggested to increase the half-life of Hsp70-substrate complexes (Höhfeld et al. 1995). Finally, CHIP (Ballinger et al. 1999) acts as an E3-ubiquitin ligase that ubiquitinates HSPA8/Hsc70/Hsp73 (i.e. the constitutive cytosolic Hsp70) substrates, promoting their degradation by the proteasome (Höhfeld et al. 2001).

## 2. Links between Hsp70 and the pathogenesis of PD

PD is currently thought to involve different pathogenic mechanisms that eventually lead to neurodegeneration, as discussed elsewhere in this book series. There is substantial evidence supporting a prominent role in PD-related cell death of aSyn toxic oligomers. One hypothesis postulates that certain aSyn species can affect the homeostasis of cell membranes (Gupta et al. 2008; Lashuel, Petre, et al. 2002; Volles & Lansbury 2002), and produce ER and oxidative stress, UPS and mitochondrial dysfunction (Gupta et al. 2008; Jellinger 2010), as well as neuroinflammation (Jellinger 2010; Roodveldt et al. 2008) processes, all of which have been linked, to a lower or larger extent, to the amyloid-like aggregation of aSyn. As will be described below, the Hsp70 system has been found to be a key player in counteracting most of these processes, not only by physically interacting with aSyn, but also by promoting aggregation clearance

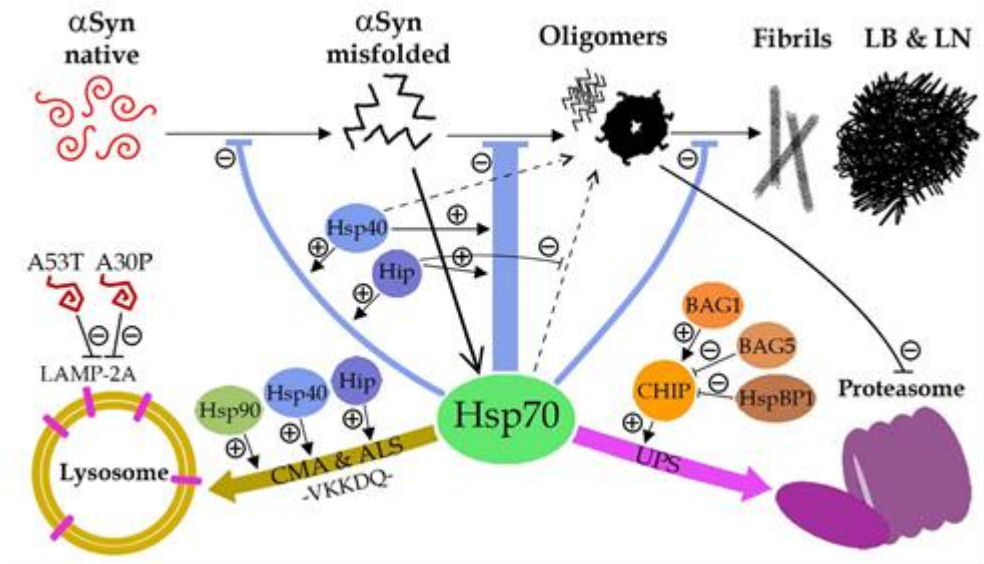


**Fig. 1. Links between Hsp70 and the multiple aSyn-mediated processes in PD pathogenesis.** CMA: chaperone-mediated autophagy; UPS: ubiquitin-proteasome

system; LB: Lewy bodies; LN: Lewy neurites. Discontinuous lines depict the possible 'sequestration' of certain chaperones by aSyn aggregating species

## 2.1 Hsp70 in modulation of aSyn aggregation and cytotoxicity

Heat-shock proteins (HSPs) prevent and reverse the misfolding and aggregation of proteins, and the Hsp70 family in particular is known to play important roles in protecting neurons from protein aggregation-derived stress (Lu et al. 2010). Therefore, it might not seem surprising that Hsp70 has been found to be linked to several neurodegenerative processes and 'conformational' disorders, including PD (Witt 2010). Notably, Hsp70 has been shown to colocalize with aggregated aSyn within LBs in brains from PD patients (Auluck et al. 2002), strongly suggesting a role for this chaperone in managing aSyn aggregates in the context of PD (Figure 1). It is then that a substantial portion of the research in the field has focused on the effects of this major cytosolic chaperone on aSyn aggregation and cytotoxicity. Following the discovery that Hsp70 can abrogate the neurotoxicity of abnormal polyglutamine proteins (Warrick et al. 1999), it was shown that Hsp70 can also prevent dopaminergic neuronal loss associated with aSyn in a Drosophila PD model (Auluck et al. 2002). Numerous studies that followed have reported that over-expression of Hsp70 is able to reduce aSyn aggregation and/or toxicity in various cellular models (Danzer et al. 2011; Klucken, Shin, Masliah, et al. 2004; McLean et al. 2004; Opazo et al. 2008; Outeiro et al. 2008; Zhou et al. 2004). In particular, McLean and co-workers (Outeiro et al. 2008) have found that Hsp70 rescues aSyn-linked toxicity by promoting the cellular clearance of aSyn oligomers, rather than monomers. Another study (Opazo et al. 2008) found that Hsp70 manages aSyn intracellular aggregation by increasing the clearing of aggregates primarily through the aggresome, and the subsequent removal of small aggregates and aggresomes from the cytosol. An interesting study by McLean and colleagues (Danzer et al.

2011) has recently shown that Hsp70 can also inhibit the formation of extracellular aSyn oligomers and rescue the cytotoxicity produced by such secreted oligomers, in a cellular model. Moreover, their data also indicates that Hsp70 is released to the extracellular medium together with secreted aSyn, adding to the accumulating evidence that Hsp70 can be released from cells by an active mechanism, with functionally relevant consequences.

Intriguingly, while it was found that over-expression of Hsp70 can prevent aSyn aggregation in a Wt aSyn transgenic mouse model (Klucken, Shin, Masliah, et al. 2004), a recent work based on A53T-aSyn transgenic mice has failed to observe this effect (Shimshek et al. 2010), seeding some controversy. Could these findings reflect a difference in the nature of the aggregates generated by Wt and mutant aSyn, and therefore a differential ability of Hsp70 to cope with those aggregates? Even though there is compelling evidence demonstrating the important role of Hsp70 under physiological and pathological scenarios in modulating fibril formation based on in vivo and cellular models, the molecular mechanism underlying such anti-aggregation properties in the context of PD, is still not fully understood.

Unlike the many research works performed with PD cellular and animal models that have contributed to our understanding on Hsp70 function under physiological or pathological conditions, only a handful of studies have focused on the molecular mechanism and interactions that underlie the modulation of aSyn aggregation exerted by Hsp70. An in-cell fluorescence resonance energy transfer (FRET) study indicated that Hsp70 alters the conformation of aSyn, inducing it to adopt a more 'open' conformation, without affecting the aSyn-aSyn intermolecular interactions (Klucken et al. 2006). Similarly to what had been found in vitro for Hsp70 and the HD-related huntingtin protein (Muchowski et al. 2000; Wacker et al. 2004), several studies with aSyn have

shown that Hsp70 is able to suppress aSyn fibril assembly. In this case, a variety of in vitro studies have shown that this efficient inhibition of amyloid assembly can occur in the absence of collaborating co-chaperones and in an ATP-independent manner (Ahmad 2010; Dedmon et al. 2005; Huang et al. 2006; Luk et al. 2008; Roodveldt et al. 2009), while promoting formation of small oligomeric species of moderate toxicity (Roodveldt et al. 2009). However, our recent study demonstrates that, even though Hsp70 is indeed able to control aSyn fibrillation and its associated toxicity in an ATP-independent manner, it does so more efficiently in the presence of ATP (Roodveldt et al. 2009), consistent with the results obtained with a cellular model (Klucken, Shin, Hyman, et al. 2004). Nevertheless, this ATP-dependent activity was found to require Hip co-chaperone activity, and not Hsp40 as observed in the case of mutant huntingtin protein (Lotz et al. 2010), suggesting that Hsp70 can function through different molecular mechanisms, depending on the nature of the aggregating client protein.

Interestingly, our study also showed that Hsp70 in the presence of ATP is prone to coaggregate with aggregating aSyn, presumably caused by the formation of a highly insoluble (ADP)Hsp70/aSyn complex. Surprisingly, this co-aggregation can be prevented by the addition of the co-chaperone Hip (St13), which had been found to be under-expressed in serum of PD patients since the early stages of the disease (Scherzer et al. 2007). The relevance of Hip in assisting the suppression of aSyn aggregation in an Hsp70-dependent manner was further supported by a study we performed in a C. elegans model of PD (Roodveldt et al. 2009), in which knock-down of Hip produced a much stronger PD phenotype than knock-down of Hsp70, shown by higher levels of aSyn aggregation in the former condition. This finding indicates that Hip co-chaperone could be important not only in modulating the chaperone's molecular mechanism, but

also in guarding the functionality and availability of Hsp70 under certain conditions.

One relevant question relates to the nature of the species along the aggregation pathway of aSyn that are specifically recognized and targeted by Hsp70. Initially, Hsp70 was reported to bind to aSyn filaments in vitro (Lindersson et al. 2004). However, it is currently understood that Hsp70 does not disaggregate or alter the structural properties of mature aSyn fibrils (Dedmon et al. 2005), but rather inhibits fibril formation via interactions with soluble pre-fibrillar forms of aSyn. A few studies performed with cell extracts (Zhou et al. 2004) and with live cells (Klucken et al. 2006), in addition to experiments with purified proteins (Dedmon et al. 2005; Huang et al. 2006; Roodveldt et al. 2009), demonstrate the existence of molecular interactions between Hsp70 and aSyn oligomeric species. Even though previous attempts to isolate such complexes by co-immunoprecipitation or pulldown experiments had failed (Luk et al. 2008), we have been able to probe the formation of a complex between Hsp70 and aSyn oligomeric species by FRET (Roodveldt et al. 2009), suggesting that these interactions are transient (Luk et al. 2008; Roodveldt et al. 2009), besides being highly dynamic (Luk et al. 2008; Roodveldt et al. 2009).

In addition, we have recently shown by FRET and nuclear magnetic resonance (NMR) analyses that, contrary to what was previously thought, Hsp70 can also interact with aSyn monomeric species (Roodveldt et al. 2009) with an affinity constant lying within the low micromolar range. Moreover, the fluorimetric study revealed the existence of diverse Hsp70/aSyn complexes that are formed depending on the nucleotide state of the chaperone, either nucleotide-free, or ATP- or ADP-bound, that are sampled along the ATPase cycle. Based on these structural studies, we have proposed that a particularly 'compact' Hsp70/aSyn

complex in the ADP-state of Hsp70 that arises during the aggregation process of aSyn, leads to Hsp70 being entrapped or sequestered by the oligomers. However, we propose that this compact complex can be stabilized by the Hip co-chaperone and therefore the co-aggregation of the chaperone, and ultimately the formation of aSyn fibrils, can be prevented (Roodveldt et al., 2009). In other words, our findings indicated that a decreased expression of Hip could facilitate depletion of Hsp70 by amyloidogenic polypeptides, impairing chaperone proteostasis. Interestingly, another case of chaperoneinhibition/ depletion by aSyn oligomers, but not by unstructured monomers, was recently reported for the Hsp70/Hsp40 system (Hinault et al. 2010). In this case, the authors found that the inhibition of the chaperone system was predominantly caused by the sequestration or incapacitation, by off-pathway aSyn oligomers, of the J-domain (Hsp40) co-chaperone (Hinault et al. 2010).

What is the region on the aSyn molecule thought to be recognized and bound by Hsp70? One study mapped this region as the broad segment between residues 21 and 110 (Luk et al. 2008). Based on a predictive algorithm for Hsp70-binding regions (Rüdiger et al. 1997) and FRET analyses to probe Hsp70-aSyn interactions (Roodveldt et al. 2009), our results indicate that Hsp70 can bind to the N-terminus and the central NAC region of the protein. The first binding region is involved in functional lipid interactions, while the second one comprises the stretch of hydrophobic residues that readily forms fibrils in vitro and is generally assumed to be involved in initiating the fibrillation process (Giasson et al. 2001). In addition, our results suggest that Hsp70 also interacts with the negatively charged C-terminus of the aSyn molecule, especially in the nucleotide-free state. Taken together, the studies carried out thus far demonstrate that Hsp70 modulates aSyn aggregation by interacting with the protein at different stages of aggregation, by recognizing essentially two or

three regions in the aSyn molecule, and forming different transient complexes with the substrate. The strong binding versatility displayed by Hsp70 with aggregating aSyn might be possible thanks to the large structural flexibility conferred by the lid subdomain within the SBD, that has been recently described by Mayer and colleagues (Schlecht et al. 2011).

## 2.2 Cooperation of Hsp70 with the ubiquitin-proteasome system (UPS).

### 2.2.1 Involvement of the ubiquitin-proteasome system in PD.

The ubiquitin-proteasome system (UPS) is responsible for the degradation of vital regulatory proteins that control almost every cellular function (Hershko & Ciechanover 1998). The UPS system is composed of three classes of ubiquitinating enzymes (E1, E2 and E3) that activate, transfer and attach the small protein ubiquitin to the Lys residues of proteins that are targeted for degradation. Conjugation of at least four ubiquitin moieties acts as the degradation signal in a process that is initiated by the recognition of the ubiquitin linkage by the proteasome, which first catalyzes the unfolding and then the proteolysis of the targeted protein function (Hershko & Ciechanover 1998).

Impairment in the UPS has been linked to many neurodegenerative disorders and indeed to contribute to disease progression in PD (Ciechanover & Brundin 2003; Cook & Petrucelli 2009; McNaught et al. 2001). The involvement of UPS in PD arouse major interest with the identification of mutations in the E3 ubiquitin ligase parkin protein as a cause of autosomal recessive PD (Kitada et al. 1998). Both the loss of E3 activity and the possibility of incomplete or aberrant ubiquitination are proposed as causes of parkin-related PD (Giasson & Lee 2003). A second member of the UPS involved in PD is the ubiquitin carboxy-

terminal hydrolase-L1 (UCH-L1), and mutations in the uchl-1 gene cause dysfunction of this enzyme and lead to accumulation of toxic products (Leroy et al. 1998).

Beyond the clear genetic association between the UPS and PD, several studies have demonstrated a reduced proteolytic activity in the SN of PD patients when compared with aged-matched controls (McNaught et al. 2003; McNaught & Jenner 2001; McNaught & Olanow 2006). In culture of rat primary neurons, treatment with proteasomal inhibitors has shown to lead to the formation of aSyn- and ubiquitin- positive proteinaceous inclusions, analogous to LBs found in PD patients (McNaught, Mytilineou, et al. 2002; Rideout et al. 2005). Moreover, systemic administration of proteasomal inhibitors in rats produced a behavioural and pathological phenotype strongly reminiscent of PD (McNaught et al. 2004), although recently some controversy arose in this matter (Manning-Boğ et al. 2006). Further support to the active role played by proteasomal impairment in PD progression originated from studies with pesticides, such as rotenone and paraquat (Cook & Petrucelli 2009). Mice treated with such environmental toxins display a strong reduction in proteolytic activity that is dependent on the presence of aSyn (Fornai et al. 2005). All together, these data undoubtedly link aSyn aggregation with impairment of the UPS in PD progression.

In relation to protein misfolding and aggregation, it has been proposed that failure of the UPS to adequately remove misfolded or abnormal proteins may underlie demise of nigral cells in sporadic PD (McNaught et al. 2001). Furthermore, deficits in the 26/20S proteasome pathways are accompanied by protein accumulation and aggregation, which may also cause neurodegeneration (Chung et al. 2001), in line with recent findings that general intracellular aggregation of proteins into aggresomes can inhibit the UPS

(Bence et al. 2001). Moreover, chaperones of the heat-shock families, including HSP27, 40, 70, 60, 90, and 110, as well as components of the UPS, such as ubiquitin, UCH-L1 and parkin, are found in LBs extracted from PD patients post mortem (Shults 2006). These combined pieces of evidence have attracted much attention lately as they imply that LBs could originate from ubiquitin-rich aggresomes that the proteasomal components may not be able to process (McNaught, Shashidharan, et al. 2002). One plausible mechanism would involve aSyn adopting abnormal protein conformations and overwhelming the cellular protein degradation systems (Wong & Cuervo 2010), whereas deficits in the UPS machinery would challenge the cell's ability to detect and degrade misfolded proteins that can result in the formation of toxic early aggregates (McNaught, Shashidharan, et al. 2002). The common outcome of this failure at different levels is thus expected to be a cellular build-up of unwanted toxic species that should have been cleared in otherwise healthy conditions. Minimal defects in the crucial protein turnover machinery may suffice to cause a slow demise of dopaminergic neurons, which may explain the relentless, progressive nature of the disease (Vila & Przedborski 2003).

Alpha-synuclein extracted from LBs in PD brains has been found to be mono- and di-ubiquitinated (Hasegawa et al. 2002), while soluble aSyn is mono-ubiquitinated by SIAH-1 and -2, but not by parkin (Liani et al. 2004; Rott et al. 2008). Instead, a modified, Oglycosylated, version of aSyn is a substrate for parkin-induced ubiquitination (Shimura et al. 2001), and it was shown that interaction of parkin with aSyn is mediated by synphilin-1 (Chung et al. 2001) as well as by the protein 14-3-3-η (Sato et al. 2006). Importantly, parkin has been shown to be able to rescue primary neurons from the toxic effects of aSyn (Petrucelli et al. 2002), suggesting that the two proteins share a common pathway that may determine the fate of dopaminergic neurons in PD. In

addition, UCH-L1 may be involved in regulating the cytoplasmic abundance of aSyn, as it displays unexpected ubiquitin ligase activity that is also able to poly-ubiquitinate mono- and di-ubiquitinated aSyn (Liu et al. 2002). Since attachment of at least four ubiquitin molecules is known to be required for protein degradation via the UPS (Hershko & Ciechanover 1998), it is likely that mono-, di-, and aberrant poly-ubiquitinated aSyn could have a pathogenic impact (Rott et al. 2008; Sun & Chen 2004).

### 2.2.2 Role of the C-terminus Hsp70-interacting protein (CHIP) in PD

Hsp70 and Hsp90 family members as well as small HSPs all take part in the degradation of protein substrate and are able to cooperate with the UPS towards this goal (Patterson & Hohfeld, 2006). Notably, protein homeostasis thus appears to be tightly controlled by interplay between the protein folding and protein degradation systems. Hsp70 takes part in the degradation of immature and aberrant forms of certain proteins, particularly ER-bound membrane proteins (Taxis et al. 2003), but also some cytosolic and nuclear proteins (Bercovich et al. 1997). For example, Hsp70 assists in the folding of the aggregation prone cystic fibrosis transmembrane conductance regulator (CFTR); however, Hsp70 is also able to present CFTR to the UPS and thus control precisely the abundance of this protein known to accumulate in aggregosomes and to cause cystic fibrosis (Zhang et al. 2001). Indeed, Hsp70 is actively recruited to aggregosomes and it has been proposed that this chaperone can reduce aggregosome formation by stimulating proteasomal degradation of misfolded proteins (Dul et al. 2001; García-Mata et al. 1999). The proposed mechanism for chaperone-UPS cooperation is that both systems compete for the same misfolded and aggregation-prone substrate proteins, and that the efficiency of chaperones in maintaining these proteins in solution increments

the probability of the UPS to degrade aberrant polypeptides. Alternatively, failure of chaperones to keep misfolded proteins in a soluble state gives rise to aggregates that are not efficiently degraded by the UPS and, moreover, may inhibit UPS activity towards other protein targets, altering protein homeostasis (**Figure 1**).

Insights into the mechanism that enables the cooperation of protein chaperones with the UPS have been obtained from the identification and functional characterization of the C-terminal Hsp70 interacting protein (CHIP) co-chaperone (Ballinger et al. 1999; Höhfeld et al. 2001; McDonough & Patterson 2003). CHIP contains three tetratricopeptide repeat domains (TPR) that recognize and bind the EEVD motif in both Hsp70 and Hsp90 (Ballinger et al. 1999). CHIP-complexed Hsp70 displays reduced ATP hydrolysis in vitro, suggesting that the co-chaperone diminishes the on-rate of binding and release cycles in Hsp70 (Ballinger et al. 1999). In its C-terminus, CHIP possesses an ubiquitin ligase domain (U box) that is capable of targeting proteins to degradation, in a homologous manner to RING finger domains found in E3 ubiquitin ligase enzymes (Connell et al. 2001; Meacham et al. 2001).

CHIP has been shown to efficiently act as E3 ligase for several Hsp70 and Hsp90 substrates, such as the glucocorticoid receptor and Erb2 (Dickey et al. 2007; McDonough & Patterson 2003; Murata et al. 2001). Proteins ubiquitinated by CHIP are efficiently targeted to the proteasome and subsequently degraded. Notably, the UbcH4/UbcH5 proteins are E2 conjugating enzymes (Demand et al. 2001) that are stress-activated, suggesting that upon stress CHIP furnishes the cell with chaperone-dependent ubiquitin ligases capable of ubiquitinating misfolded and aggregation-prone substrates.

The ubiquitinating activity of CHIP is regulated by two co-chaperones, BAG-1, which is an enhancer, and the Hsp70 binding protein 1 (HspBP1), which acts as a repressor. The co-chaperone BAG-1 associates with the ATPase domain of Hsp70 and, in addition, possesses a ubiquitin-like domain that is efficiently recognized and bound by the proteasome (Lüders et al. 2000). Thus, BAG-1 provides the proteasome with the capability to recruit Hsp70 and consequently degrade its cargo clients. A ternary complex involving BAG-1, Hsp70 and CHIP has been isolated, and BAG-1 is capable of stimulating CHIP mediated degradation of some proteins (Alberti et al. 2002). The co-chaperone HspBP1, in turn, competes with BAG-1 for binding to the ATPase domain of Hsp70 (Alberti et al. 2004). Notably, when in complex with HspBP1 bound Hsp70, CHIP shows a much reduced ubiquitin ligase activity and chaperone substrates are no longer ubiquitinated and target to the UPS (Alberti et al. 2004).

In the context of neurodegeneration, CHIP has been shown to intervene in the degradation of misfolded aggregation-prone proteins associated with AD, PD and HD, as well as with spinocerebellar ataxia and spinal bulbar muscle atrophy (Adachi et al. 2007; Al-Ramahi et al. 2006; Hatakeyama et al. 2004; Shin et al. 2005; Urushitani et al. 2004). Indeed, CHIP plays an active role in modulating aSyn aggregation and degradation (Kalia et al. 2011; Shin et al. 2005; Tetzlaff et al. 2008). It was shown that CHIP interacts with both soluble and aggregated aSyn, and its over-expression reduces aSyn abundance and aggregation (Shin et al. 2005). Interestingly, CHIP induces aSyn clearance via two alternative pathways, one involving Hsp70 and proteasomal degradation, and another Hsp70-independent route that targets aSyn to lysosomes (Shin et al. 2005). A fluorescence-complementation assay demonstrated that CHIP reduced significantly the abundance of aSyn toxic oligomers in cell culture, suggesting that this aberrant species is preferentially recognized by the co-chaperone

(Tetzlaff et al. 2008). More recently, it was discovered that CHIP is an E3 ubiquitin ligase of aSyn since it efficiently conjugates ubiquitin moieties to this protein (Kalia et al. 2011). CHIP-catalyzed ubiquitinated forms of aSyn include mono- and poly-ubiquitinated species, and the activity of CHIP depends on the presence of Hsp70 and the co-chaperone BAG5. Contrary to the enhancer activity reported for BAG1, BAG5 reduces the ability of CHIP to ubiquitinate aSyn in and Hsp70-dependent manner (Kalia et al. 2011).

Additional pathogenic mechanisms in PD that involve the activity of CHIP relate to the ubiquitin ligase activity of parkin and the kinase activity of the leucine-rich repeat kinase-2 (LRRK2). It has been found that CHIP, Hsp70 and parkin form a ternary complex that promotes ubiquitination and degradation of the Pael receptor, a protein localized in the ER and whose accumulation has been linked to dopaminergic neuronal death (Imai et al. 2002). CHIP is proposed to enhance the activity of parkin, even in the absence of Hsp70 (Imai et al. 2002). Concerning LRRK2, CHIP regulates the ubiquitination, degradation, and toxicity mediated by pathogenic mutations of this kinase (Ko et al. 2009). CHIP binds LRRK2 via its TPR motifs and formation of this complex is protective in cell culture models, while in the presence of mutant LRKK2, knock-down of CHIP leads to cell death (Ko et al. 2009).

### 2.3 Hsp70 in chaperone-mediated autophagy (CMA)

Macroautophay and chaperone-mediated autophagy (CMA) are the two main lysosomal proteolytic systems in mammalian cells for the degradation of intracellular proteins (Xilouri & Stefanis 2011). CMA is the process of degradation of intracellular components by lysosomes which selectively degrades cytotolic proteins containing a KFERQ-like motif (Koga & Cuervo 2011). This process is known to involve binding of a complex of constitutive

cytosolic Hsp70 (Hsc70) and co-chaperones (including Hsp40, Hip, Hsp90 and BAG1) to substrate proteins, and their subsequent targeting to lysosomes via the lysosomal surface receptor LAMP-2A (Xilouri & Stefanis 2011). The substrate protein is subsequently degraded after unfolding and translocation into the lysosomal lumen, in a process involving lysosomal Hsc70 (**Figure 1**). It has been estimated that about 30% of cytosolic proteins could be subjected to degradation via CMA (Dice 2007), and furthermore, this pathway may be a major route by which aSyn is degraded in neurons (Witt 2010). Even though it is currently accepted that dysregulation of autophagy plays a role in neurodegeneration (Bandhyopadhyay & Cuervo 2007; Nixon 2006; Rubinsztein 2006; Xilouri & Stefanis 2011), including the PD neurodegenerative process (Martinez-Vicente et al. 2008; Yang et al. 2009), the mechanism by which CMA modulates neuronal survival or death, is still unclear.

Given that pathologic accumulation of aSyn is a hallmark of PD, several recent studies have addressed the possible link between aSyn degradation, CMA dysfunction and the neurodegenerative process. Indeed, aSyn, which contains a pentapeptide sequence ($_{95}$VKKDQ$_{99}$) consistent with Hsc70 binding (Dice 1990), has been shown to be degraded via CMA using isolated lysosomal preparations (Cuervo et al. 2004) and neuronal cells (Alvarez-Erviti et al. 2010; Martinez-Vicente et al. 2010; Vogiatzi et al. 2008). On the contrary, the A30P and A53T aSyn variants were observed to bind strongly to LAMP-2A receptors but were not internalized, thus inhibiting the CMA degradation of other substrates (Cuervo et al. 2004). This CMA dysfunction was later shown to mediate aSyn toxicity in cellular models (Xilouri et al. 2009). Moreover, a recent study using both aSyn transgenic- and paraquat- PD mouse models (Mak et al. 2010), has shown that aSyn can be degraded in the lysosome through CMA, *in vivo*. The study also revealed an up-regulation of LAMP-2A and lysosomal Hsc70 and an

increase in Hsc70-aSyn interactions in brain lysosomes, relative to controls. On the other hand, a recent work showed a significant reduction of both LAMP-2A and Hsc70 levels in the SN and amygdala of PD brains, relative to age-matched Alzheimer's disease (AD) and healthy, brain controls (Alvarez-Erviti et al. 2010). Even though their results might initially appear contradictory, these findings support a key role for Hsc70 and the CMA system in maintaining intracellular general proteostasis, especially within the aSyn-overload scenario that is typically associated to PD and other α-synucleinopathies.

## 2.4 Emerging links between the Hsp70 system and neurodegeneration in PD

### 2.4.1 The CSPα-Hsc70-SGT complex and neurodegeneration in PD

Cysteine-string protein α (CSPα) is an abundant protein localized in synaptic vesicles that maeliorates neurodegeneration in cellular and animal models (Johnson et al. 2010). It contains a Dna-J domain and has been shown to interact with Hsc70 and to increase its ATPase activity (Braun et al. 1996). CSPα has been shown to assemble into an enzymatically active ternary complex with Hsc70 and SGT (small glutamine-rich tetratricopeptide repeat domain protein) with a likely regulatory function in secretory vesicles (Tobaben et al. 2001). CSPα has also been reported to interact with other chaperones, including Hsp90 (Sakisaka et al. 2002), Hip, Hop (Rosales-Hernandez et al. 2009), and Hsp40 (Gibbs et al. 2009).

CSPα dysfunction has been implicated in various pathologies, including memory impairment, type-2 diabetes, cystic fibrosis, and HD (reviewed in (Johnson et al. 2010)). A few years ago, a link between CSPα and PD was also established (Chandra et al. 2005). The results of this study, performed using a transgenic mouse model, indicate that aSyn cooperates with CSPα in preventing

neurodegeneration (Chandra et al. 2005). In a recent study (Sharma et al. 2011), the CSPα-Hsc70-SGT complex was found to bind to monomeric SNAP-25, a pre-synaptic SNARE protein, and prevent its aggregation, thus promoting the formation of the SNARE complex involved in neuronal synapse. Interestingly, another recent study using a transgenic mouse model of aSyn aggregation with associated neurodegeneration, has shown that SNAP-25 is redistributed within synaptic terminals and the protein was found to colocalize with aSyn within intraneuronal aggregates (Garcia-Reitböck et al. 2010). These data raise the question whether aggregating aSyn might actually be sequestering CSPα involved in the CSPα-Hsc70-SGT complex, analogously to what was observed for aggregating huntingtin (Miller et al. 2003), and therefore enabling the aggregation of SNAP25 in a PD scenario.

### 2.4.2 The unfolded protein response (UPR) in PD

The unfolded protein response (UPR) is a mechanism activated within the cell when the endoplasmic reticulum (ER) function is impaired and, as a result, unfolded proteins accumulate in the ER lumen (a process called 'ER stress'). The ER-resident Hsp70 family member GRP74/BiP recognizes and binds such unfolded proteins, which causes the release UPR activating factors (Rutkowski & Kaufman 2004). To restore ER function, the UPR reduces protein translation and enhances folding and processing capacity within ER. However, if the stress overwhelms the cell restoring capacity, the UPR induces apoptosis (Paschen & Mengesdorf 2005). A few years ago, a mutation in the parkin gene was found to be related to familiar PD due to the impairment of its ubiquitin ligase function which results in protein accumulation within the ER lumen and leads to ER stress and apoptosis (Imai et al. 2001). More recently, a strong correlation between UPR activation and PD pathogenesis in PD patients was established (Hoozemans et al. 2007), with results that might suggest a functional

connection between aSyn and ER stress. In addition, it was found that overexpression of Wt aSyn triggers UPR in yeast (Cooper et al. 2006). A recent study has shown that aSyn is also found aggregated and accumulated within the ER lumen and induces UPR by binding to GRP78/BiP in cells, which could lead to apoptosis (Bellucci et al. 2011). Clearly, supplementary investigations are needed to determine the exact role of the UPR in the pathophysiology of PD.

### 2.4.3 Mitochondrial dysfunction in PD

In both familial and sporadic forms of PD, several mitochondrial alterations and increase of oxidative species are well recorded (Jellinger 2010). The important role of mitochondrial pathology in PD is reflected by the specific and selective loss of mitochondrial complex I activity in the SN of PD patients (Valente et al. 2004). Results from a study with neuronal cell cultures indicated that this impairment was dependent on aSyn mitochondrial import and accumulation (Devi et al. 2008). Interestingly, a recent proteomic study revealed that expression of mortalin (mtHSP70/GRP75), a mitochondrial stress protein and member of the Hsp70 chaperone family which binds to DJ-1 and aSyn (Jin et al. 2007), is significantly decreased in PD brains (De Mena et al. 2009; Jin et al. 2006) as well as in a cellular model of PD (Jin et al. 2006). Moreover, specific coding mutational variants of the mortalin gene have been recently discovered in a few PD patients (Burbulla et al. 2010; De Mena et al. 2009). Finally, differential levels of mitochondrial mortalin were measured in Wt and A53T cellular models of PD (Pennington et al. 2010), suggesting a possible involvement of aSyn aggregation in PD-related mitochondrial dysfunction (Xie et al. 2010).

## 3. Hsp70 machinery members as biomarkers in PD

A biomarker is a naturally occurring molecule, gene, or characteristic by which a particular medical condition, disease, etc. can be identified. Despite the current relevance of identifying a biomarker for early diagnosis of PD and/or to follow up its progression, up to date there is no reliable biomarker available (Morgan et al. 2010; Nyhlén et al. 2010). Therefore, certain key proteins that are tightly linked to PD pathogenesis or progression, such as the members of the Hsp70 machinery discussed above, which could manifest changes in their expression levels in body fluids cells or alter their presence in body fluids in a PD scenario, could represent potential markers of disease development or predisposition.

Nowadays the most promising biomarkers for PD in cerebrospinal fluidic (CSF) are aSyn, DJ-1, amyloid β, and the tau protein. These are the principal targets in the Parkinson's Progression Markers Initiative, a public-private, large-scale study project that aims to identify biological markers of disease progression spearheaded by the Michael J. Fox Foundation (www.PPMI-info.org). DJ-1, which is the only chaperone to be included in this study, is a mitochondrial chaperone which has been one of the most studied proteins for its potential use as a PD biomarker. However, results published thus far from measurements of DJ-1 in CSF (Hong et al. 2010) and serum from PD patients (Hong et al. 2010; Shi et al. 2010; Waragai et al. 2007) are somewhat controversial or inconsistent, which could probably be explained by the high DJ-1 protein level present in blood cells (Shi et al. 2010).

Currently, it is well established that certain proteins, including members of the Hsp70 family such as Hsp701A and 1B, display perturbed expression levels in the SN of PD brains (Hauser et al. 2005); however, changes in tissue expression levels are in principle not useful for an application as biomarkers. Recently, a significant decrease in whole blood mRNA levels of St13/Hip co-chaperone was reported for early PD patients, but not for AD patients or healthy controls

(Scherzer et al. 2007). A second group later found differences in HSPA8 (Hsc70) and HIP2 expression levels between PD patients and controls (Grünblatt et al. 2010). However, a third study reported no significant differences in the expression pattern of ST13 in early-stage PD patients, as compared to controls (Shadrina et al. 2010). This discrepancy could be attributed, at least in part, to heterogeneity in the criteria of diagnosing and classifying the individuals into groups and to the difficulty in establishing the actual onset of the disease. Other members of the Hsp70 machinery are known to change their expression patterns in PD patients compared to healthy controls (Hauser et al. 2005). Unfortunately, these changes appear not specific or sufficient to differentiate between PD and other related neurodegenerative disorders (Hauser et al. 2005). This is probably due to the fact that the Hsp70 machinery plays a central role in maintaining cell proteostasis, which is perturbed in a variety of neurodegenerative diseases.

Considering that PD is a complex pathology that involves several systems such as the stress response, the UPS, the immune system, etc., a unique biomarker might not be enough as a tool for diagnosis or follow-up of disease progression. Instead, there is general consensus that the use of a set of distinct parameters, such as protein expression profiles, age, symptoms and others, would probably be the best approach (Fasano et al. 2008; Grünblatt et al. 2010; Scherzer et al. 2007).

## 4. The Hsp70 system as a therapeutic tool for PD

As described in this chapter, in the last few years it has become evident that HSPs play an important role in the initiation and progression of PD and other neurodegenerative diseases. This cumulative evidence has prompted the development of therapeutic tools based on the Hsp70 machinery. Different

strategies have been tested to manipulate Hsp70 as a therapeutic approximation for PD and related neurodegenerative diseases (reviewed in (Kalia et al. 2010)). Three general approaches have been explored, namely, to increase the intracellular activity of Hsp70, to overexpress Hsp70 and/or other co-chaperones, and to deliver chaperones or regulatory factors using cell-penetrating peptides (CPPs).

### 4.1 Increasing the intracelular activity of Hsp70

Theoretically, it should be feasible to control the activity of the cellular chaperone machinery by using different types of drugs. The mechanism of most of such chemical compounds is based on activating HSF-1, a key transcriptional regulator of the heat shock response (HSR) that activates the gene expression of inducible HSPs. One way of activating HSF-1 is by inhibiting Hsp90 activity; as a result, HSF-1 becomes active and increases the expression of inducible chaperones like HSPA1A/Hsp72 and others. Geldanamycin (GA) and its derivative 17-allylamino-17-demethoxygeldanamycin (17-AAG) are antibiotics with high affinity for the ATPase domain of Hsp90, which blocks its interaction with HSF-1 and subsequently allows its activation (Waza et al. 2006; Zou et al. 1998). GA prevents aSyn induced dopaminergic cell loss in cell culture (McLean et al. 2004) and in animal models of PD (Auluck et al. 2005; Shen et al. 2005), while the less toxic 17-AAG has also proven to be neuroprotective in PD cellular models (Danzer et al. 2011; Riedel et al. 2010) and in two animal models of PD-related neurodegenerative diseases (Fujikake et al. 2008; Waza et al. 2005). Even though 17-AAG is currently under phase-II clinical trials as an anti-tumour drug (Pacey et al. 2012; Richardson et al. 2010; Solit et al. 2008), its use in patients with neurodegenerative diseases could be hampered by its toxicity and unavailability for oral administration (Pacey et al. 2012). Another family of inhibitors of Hsp90 activity is SNX- 2112 and its analogues, which are orally

available and present improved blood brain barrier (BBB) permeability. In particular, SNX-0723 was shown to prevent aSyn oligomer formation and aSyn-induced toxicity in cell culture (Putcha et al. 2010), and preclinical studies for cancer therapy are proving their safety (Zhai et al. 2011), although similar studies in neurodegenerative animal models are still needed.

Other drugs are able to activate HSF-1 without inhibiting Hsp90 activity, which could represent a less toxic approximation for neurodegenerative pathologies. Arimoclomol, for example, has already been tested in phase I- and IIa-clinical trials for treating ALS, and shown to be safe and tolerable (Cudkowicz et al. 2008; Phukan 2010). Another example is HSF-1A (Neef et al. 2010), shown to upregulate Hsp70 expression and to reduce poly-Q cytotoxicity in cell and fly models of poly-Q neurodegenerative disorders (Neef et al. 2010). Celastrol, yet a similar drug, and some structural relatives, appear as promising drugs due to their rapid kinetics and low EC50 (Westerheide et al. 2004), although further studies are needed.

Besides these approaches, HSF-1 co-inducers could represent more tolerable drugs for therapy. These are molecules that partially activate HSF-1, reducing its activation threshold and often working in conjunction with secondary stress signals to fully induce HSR. Non-steroidal anti- inflammatory drugs (NSAIDs) are well known co-inducers of HSR. For example, Sodium salicylate and Indomethacin induce HSF-1 DNA binding and reduce the temperature required for triggering the HSR (Jurivich et al. 1995; Lee et al. 1995). The association of NSAIDs use with a lower risk of common neurodegenerative diseases such as AD and PD has been analysed in several studies (Etminan et al. 2008; Gagne & Power 2010). According to one hypothesis, this negative correlation could be due to continuous up-regulation of HSR and consequently continued cytoprotection against neurodegenereration (Westerheide et al. 2004).

### 4.2 Overexpression of Hsp70 and/or related co-chaperones

In principle, it should be possible to design a gene therapy approach for the treatment of PD and other conformational neurodegenerative diseases based on HSPs, considering the substantial number of reports having characterized the molecular pathways by which Hsp70 acts in the context of disease. Intriguingly, even though overexpression of Hsp70 has been shown to be protective in animal models of PD (Dong et al. 2005; Jung et al. 2008), a recent study in mouse indicates otherwise (Shimshek et al. 2010). Up to date, one phase-I study in PD patients using recombinant Adeno-Associated Virus (AAV) to deliver aromatic aminoacid decarboxylase enzyme into the putamen, supports the proof-of-principle for the use of gene therapy in PD (Christine et al. 2009). With this precedent, gene therapy could potentially be employed to overexpress other chaperones and co-chaperones that may improve Hsp70 function and its neuroprotective properties. Indeed, recombinant AAV has been already used to transduce Hsp104 (Vashist et al. 2010), a non-mammalian chaperone, in a rat model of PD and proven to be neuroprotective by disaggregating protein inclusions and synergizing with endogenous Hsp70 (Lo Bianco et al. 2008).

Given that co-chaperone BAG-5 is known as a negative regulator of Hsp70, downregulation of its expression has been tested in a mouse model of PD. In this study, direct expression in the SN by recombinant AAV delivery of BAG-5(DARA), a BAG-5 mutant which inhibits wild-type BAG-5 activity, resulted in increased dopaminergic neuron survival (Kalia et al. 2004). Another possible approach to increase Hsp70 function by using gene therapy techniques could be gene silencing by RNA interference to knockdown Hsp70 downregulators. Although these techniques have not been extensively tested for

neurodegenerative therapy, they remain a potentially useful tool (Manfredsson et al. 2006).

### 4.3 Chaperones or regulatory factors delivery using CPPs

Cell penetrating peptides (CPPs) are the most recent approximation that shows promise towards increasing Hsp70 activity within cells. These are peptide motifs that allow cell transduction of macromolecules including functional full-length proteins. The basic domain of the trans-activator of transcription (TAT) from HIV-1 is the best known among CPPs and it has been shown that fusion with TAT allows proteins to penetrate cell membranes of several cell types and even to cross the BBB (Fawell et al. 1994; Schwarze et al. 1999). In the last few years, TAT-Hsp70 transduction has been reported to be neuroprotective against different kinds of stress in cell models (Lai et al. 2005; Nagel et al. 2008) as well as in a MPTP mouse model of PD (Nagel et al. 2008). In addition, transduction of TAT-Hsp40 has also been shown to be cytoprotective against oxidative stress in cells (Kim et al. 2008). Finally, transduction of HSF-1(+)-TAT, an HSF-1 mutant fused to TAT, capable of activating HSR by itself, was demonstrated to induce to induce Hsp70 expression and to protect cells against heat stress in vitro (Hou & Zou 2009). Although further investigation is needed, CPPs tagging could prove a powerful tool in therapy against neurodegenerative diseases by allowing the efficient transduction of cytoprotective proteins and factors.

## 5. Conclusions

There is strong experimental support to propose the Hsp70 chaperone system as a key player in pathogenesis and progression of PD. This central role seems to be especially linked to aSyn, although alternative connections between the Hsp70 machinery and different pathogenic mechanisms underlying PD might

exist. Up to this point, there is substantial evidence supporting an active role of Hsp70 in well-established PD-related processes. First, in the inhibition or modulation of aSyn aggregation pathway that results in the formation of LBs and the suppression of aSyn-mediated toxicity to cells that leads to neurodegeneration. Second, a prominent role in the control of the activity of the UPS machinery, the general protein degradation and disposal system in the cell. And third, a central role in CMA, which handles the lysosomal degradation of selected cytosolic proteins, including aSyn. In addition, emerging mechanisms for Hsp70 in relation to PD include its participation in the CSPα-Hsc70-SGT complex in the neuron, and the increasing attention paid to the UPR and mitochondrial dysfunction processes in PD, both of which rely on Hsp70.

The accumulated studies thus far suggest that Hsp70 chaperone is a highly versatile protein whose anti-aggregation activity seems to involve different interactions and the formation of transient and highly dynamic complexes with various aSyn species, presumably early oligomers and probably monomers, along the aggregation pathway. This activity can be certainly modulated by the presence of nucleotides and by certain co-chaperones, in particular, Hip. Clearly, further in vitro and in cell studies with Hsp70 and co-chaperones, to better understand the full molecular mechanism of mammalian Hsp70 in managing aSyn aggregation, are needed.

As a biomarker of PD, Hsp70 seems not to represent a good candidate itself, probably due to its central role in maintaining cellular proteostasis which is perturbed in several amyloidoses and related diseases. However, the Hsp70 co-chaperone Hip might represent a potentially useful biomarker for early diagnosis of PD, although more studies are needed in this direction. Given that PD is a complex disease, a ‘complex biomarker’ (i.e. composed of various markers) appears to be the only reliable option. Finally, the Hsp70 machinery

can be indirectly enhanced by HSF-1 pharmacological activation, which represents one of the most promising therapeutic approaches for treating this complex and highly debilitating disease.

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• **Labrador-Garrido A,** Cejudo-Guillén M, Daturpalli S, Leal MM, Klippstein R, De Genst EJ, Villadiego J, Toledo-Aral JJ, Dobson CM, Jackson SE, Pozo D, Roodveldt C (**2016**) Chaperome screening leads to identification of Grp94/Gp96 and FKBP4/52 as modulators of the α-synuclein-elicited immune response". *FASEB J*. Feb;30(2):564-77.

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## PATENTS

• Combinaciones de proteínas agregantes y chaperonas moleculares para el tratamiento de proteinopatías o enfermedades conformacionales'.

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